STUDIES ON A MURINE MODEL OF TRICHOMONIASIS

BY

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To mam and dad
to say thank you

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Contents

List of abbreviations

FAC	Freund's complete adjuvant
FSH	follicle-stimulating hormone
HIHS	heat-inactivated horse serum
IgA	immunoglobulin A
IgG	immunoglobulin G
LH	leuteinising hormone
MDM	modified Diamond's medium
PBS	phosphate buffered saline
STV	SolcoTrichovac

Summary

A mouse model of trichomoniasis was developed using T. vaginalis, intravaginally inoculated into Balb/c mice. Although the infection rates obtained were not consistently, or frequently, 100%, over 2/3 of experiments yielded rates of over 50%. Estrogen-treatment of mice, prior to intravaginal inoculation with the parasites, was found to be necessary for the establishment of infection but other variables such as route, timing and amount of estrogen, diet fed to mice, age and breed of mice and variables in maintenance of the parasite line proved to be of less importance. The administration of ferric ammonium citrate, prior to infection, was found to significantly increase the infection rates.

The role of the pH of the vagina and the iron and zinc content of the vaginal secretions was also investigated. The pH of the murine vagina was found to be around neutral and this was not affected by estrogen-treatment or the presence of infection. Treatment of the mouse with iron, however, does significantly increase the vaginal pH. The iron and zinc content of the vagina was extremely low.

The importance of the murine immune response was also investigated by the use of Solco Trichovac, a vaccine in use for the treatment of trichomoniasis, and 'vaccines' prepared from parasite homogenates. Neither of these preparations appeared to have any therapeutic effect.

Overall, although this mouse model is not ideal for studying trichomoniasis, it proved to be useful in study of the vaginal microenvironment, and its relationship with the presence of T. vaginalis, and an investigation of the immune response to infection.

INTRODUCTION

1.1. THE TRICHOMONADS

1.1.1. Classification and Important Parasitic Species

Trichomonads are aerotolerant, anaerobic flagellate protozoa belonging to the order Trichomonadida, family Trichomonididae. They are classified into three genera; genus Trichomonas, genus Pentatrichomonas and genus Tritrichomonas. Classification is based mainly on the number of flagella, Tritrichomonas having three anterior flagella and Trichomonas and Pentatrichomonas having four and five respectively. Three species of trichomonad infect humans; Pentatrichomonas hominis in the intestine, Trichomonas tenax in the mouth and Trichomonas vaginalis which inhabits the urinogenital tract. T. vaginalis is the only one of the three species known to cause disease. Another trichomonad of importance is Tritrichomonas foetus which parasitises the urinogenital tract of cattle causing abortion and infertility. Tritrichomonas suis inhabits the nasal passages and large intestine of swine and has been demonstrated to be very similar to T. foetus (Hibler et al., 1960; Doran, 1957). Trichomonas gallinae and Trichomonas gallinarum both parasitise birds. Other species of trichomonads are common parasites of the intestinal tract of many animals but none have been shown to be pathogenic.

1.1.2. General Biology

Trichomonads are ovoid or pear-shaped organisms with three to five flagella and an attached recurrent flagellum that forms an undulating membrane. All flagella arise from basal bodies grouped at the anterior end just in front of the single ovoid nucleus (Honigberg and King, 1964). The nucleus contains several large electron-dense granules and the whole structure is surrounded by rough endoplasmic reticulum. The costa originates in the kinetosomal complex and extends beneath the undulating membrane. Trichomonads also have a prominant non-motile axostyle which may protrude from the posterior. Paroaxostylar bodies and paracostal bodies are found grouped around the costa and axostyle and are known as hydrogenosomes.

Reproduction is by longitudinal binary fission, nuclear division being mitotic. Only one developmental stage is known and they are not generally considered to produce cysts. They feed by pinocytosis or both pinocytosis and phagocytosis as there are no structurally differentiated organelles of feeding comparable to cytosomes.

1.1.3. Morphology of T. vaginalis

Trophozoites of T. vaginalis range from between 10 and 30 μm in length and 5 and 15 μm in width. They move rapidly with a jerky, non-directional movement. They

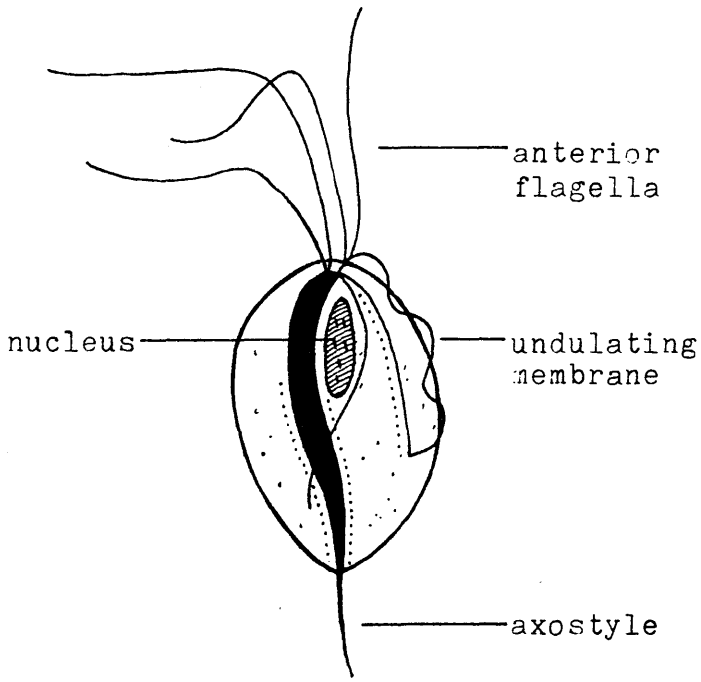
have four anterior flagella and a short undulating membrane, extending about half the distance of the body; the flagellum is not free posteriorly, unlike most other species. The undulating membrane gives a rotary motion to the body while the anterior flagella serve for propulsion. A slender rod, the axostyle, extends through the body and protrudes beyond the posterior. A long parabasal body is present, complete with parabasal filament. A costa is also present but is relatively inconspicuous, as is the cytosome. However, the nucleus is usually clearly defined and contains many chromatin granules (Fig. 1).

1.2. TRICHOMONIASIS

1.2.1. Clinical Manifestations

Clinical manifestations of trichomoniasis vary considerably between males and females and also patients of the same sex. Symptoms may also vary in intensity in a single patient, worsening and improving repeatedly. The disease is characterised, in women, by a copious, foamy yellowish-green discharge that may have a typically foul odour, as well as mild to severe vaginal itching and burning. The vaginal mucosa may be affected with pronounced hyperaemia and minute areas of haemorrhage. Additional symptoms include lower abdominal pain, painful urination and coitus, chafing of the thighs and rectal area and swelling of the lymph

Figure 1. Diagram of Trichomonas vaginalis



glands in and around the groin (Felman and Nikitas, 1979). However, as many as 25% of infected women have no symptoms at all (Felman and Wikitas, 1979).

Trichomoniasis in men is mostly asymptomatic but clinical symptoms may be like those found in gonorrhoea and non-specific urethritis and include purulent discharge, discomfort on micturition and inflammation of the external meatus. The infection can take three forms: asymptomatic, chronic and acute (Jira, 1958). The acute infection as described above can lead to chronic and latent stages and the infection can change type repeatedly. Chronic infection is characterised by a slight itching sensation inside the penis and slight moisture at the tip. Many investigators, however, think that men serve primarily as carriers, spreading symptomatic trichomoniasis among women.

1.2.2. Incidence of trichomoniasis

The apparent incidence of trichomoniasis varies greatly according to the population studied and the method of detection used. The highest rate of infection occurs with women who are at greatest risk for other venereal diseases (Felman and Nikitas, 1979), so the highest incidence is in females between the ages of 16 and 35, falling rapidly after the menopause. Cases of trichomoniasis in prepubertal girls have been recorded (Peter, 1945) but are very rare. The prevalance of

infection in the U.S.A. has been reported to range from 3 to 5% of asymptomatic women examined by private physicians to 13 to 25% of asymptomatic women examined in gynecological clinics. Among prostitutes, rates between 50 and 70% have been reported (Felman and Nikitas, 1979). Thus the overall incidence of T. vaginalis in the population of adult women probably falls between the range of 2.6% (found with a group of married women) to the 70% reported for venereal disease clinic patients (Naguib et al., 1966).

The prevalance of trichomoniasis in the male population has also been estimated and found to be 50% to 60% of the rate reported for females. Eighty per cent of male sexual partners of infected women have been found to harbour T. vaginalis, while female partners of infected males are almost always infected (Brown, 1972). The difference in reported infection rates may be due to the difficulty of detecting trichomonads in male urinogenital specimens. It has been estimated that T. vaginalis is not detected up to 50% of the time (Krieger, 1981).

1.2.3. Transmission

Transmission is generally recognised to be by sexual intercourse but it is possible that in unusual circumstances the disease may be contracted through non-venereal means. Protozoans can survive outside the body

for some time and remain active in urine for several days and in tap water for several hours (Mason, 1980), so theoretically an infection may be contracted by contact with contaminated bath or toilet articles. There is no decisive evidence that contact with contaminated articles is a source of trichomonal infection. A documented non-venereal means of transmitting infection is that of childbirth to infected mothers (Krieger, 1981) and it has been suggested that T. vaginalis can cause pneumonia in babies infected in this way (Hiemstra et al., 1984).

1.2.4. Diagnosis

Diagnosis of trichomoniasis is by demonstration of T. vaginalis in urinogenital specimens, commonly using either wet or dry smears or culture. Unfortunately, none of these methods is totally reliable. The wet smear is prepared by mixing vaginal material with a saline solution and microscopically examining it for living trichomonads, most readily identified by the typical trichomonad movement. Success with this method depends on the experience of the observer and the motility of the trichomonads (they tend to be fairly slow moving at room temperature). Various studies conducted indicate that the method is only 60-70% (Krieger, 1981) or 80% (Fouts and Kraus, 1980) accurate for women. Another method is to use dry mounts prepared using various strains. This technique is probably of

less value than wet mounts because dead trichomonads are difficult to distinguish from leucocytes, causing inaccuracy. Even culturing, the most sensitive technique may allow trichomoniasis to go undetected in ten per cent or more of asymptomatic women (Felman and Nikitas, 1979; Nielson 1973; Spence et al., 1980).

Serological detection of disease using the enzyme-linked immunosorbent assay (ELISA) has also been attempted (Street et al., 1982) but was found to be less sensitive than the culture method. A problem associated with this method is that serum antibody to T. vaginalis is found in apparently uninfected women, possibly because previous infections have resolved or there are cross-reactions with other trichomonads. Production of local antibodies appears to be of no diagnostic value (Street et al., 1982). The difficulty of using immunological methods is increased greatly by the finding of significant antigenic variations in T. vaginalis (Kott and Adler, 1961; Krieger et al., 1985; Teras et al., 1966). Recently another ELISA has been developed which detects T. vaginalis antigens present in vaginal secretions (Watt et al., 1986). Although this is not as sensitive as in vitro cultivation it is considerably more rapid. Its usefulness is yet to be established.

1.2.5. Treatment

Since the 1960's the standard treatment for

trichomoniasis has been chemotherapy using, in particular, metronidazole (manufactured in the U.K. by May and Baker under the trade name Flagyl). This is a member of a group of 5'-nitroimidazoles that also includes tinidazole and nimorazole. The 5'-nitroimidazoles are effective against anaerobic organisms. The drug enters by passive diffusion along the concentration gradient whereupon it is reduced to give a free radical. The mechanism for cytotoxicity is not understood but may involve binding to macromolecules such as DNA (Edwards 1979, 1980, 1981). The reduction of metronidazole inside the cell, under anaerobic conditions, increases the gradient of diffusion of the drug into the cell.

The efficacy of metronidazole is accepted and unsurpassed with cure rates of 95% or more using the standard dose (200 mg orally, three times a day for seven days or a single 2 g dose) (Roe, 1977). The 5% of infections that persisted in this study were possibly because of poor absorption or excessive destruction of the drug by vaginal flora (Koch-Wesler and Goldman, 1980) although metronidazole-resistant lines have been reported (Benazet and Guillaime, 1971; Meingassner et al., 1978; Lossick et al., 1986). Metronidazole's side-effects are mild, such as headache and dry mouth, but there is concern over reports of possible carcinogenic, mutagenic and teratogenic effects (Goldman, 1981).

There is also a recently introduced vaccine, SolcoTrichovac, on the market which is claimed to be highly successful in treating non-specific vaginitis and trichomoniasis (see 'SolcoTrichovac: a new concept for the treatment and prophylaxis of trichomoniasis and non-specific vaginitis'. Published by Solco Basle Ltd.). It is produced from killed variants of Lactobacillus acidophilus, isolated from the vaginal secretions of patients with trichomoniasis, and the makers suggest that mechanism of action is due to cross-reactivity between strains of L. acidophilus and T. vaginalis. An independent study of this cross-reactivity, however, failed to show any such antigenic relationship (Gombosova et al., 1986) and further investigation of the relationships between microorganisms in the vagina is required.

1.3. EXPERIMENTAL MODELS OF TRICHOMONIASIS

The study of trichomoniasis would greatly benefit from the availability of a good experimental model of the disease, that would allow investigation of fundamental aspects of the host-parasite relationship such as pathogenicity, immunology and chemotherapy. There have been numerous attempts previously to infect a variety of animals and most authors agree that transmission of T. vaginalis to laboratory animals presents difficulties.

Many workers have concentrated on intramuscular, intraperitoneal or subcutaneous routes of infection using rats (De Carneri, 1966), guinea pigs (Teras, 1954) and mice (Schnitzer et al., 1950; Teras, 1954; Ivey and Hall, 1964; Tsai and Price 1973). These models have aided studies of pathological aspects of the disease (Honigberg, 1961) but can provide little information on the natural course of infection. They have also been used extensively in studies of chemotherapy, but clearly they provide little pharmacokinetic information relevant to normal T. vaginalis infections. There is therefore a need for an intravaginal model.

There have been several reports on the experimental infection of laboratory animals by the intravaginal route, and there are some reports of success with attempts to establish intravaginal infections of monkeys (Trussel 1947; Johnson et al., 1950; Street et al., 1983). The infection rates differed between studies, with some workers reporting symptomatic infections. However, although monkeys could be a useful model for studies involving small groups of animals, they are unsuitable for most routine experiments. The infection of guinea pigs (Soszka, 1962, 1963; Kazanowska 1966; Kazanowska et al., 1973; Maestrone and Semar, 1967), rats (Cavier and Mossion, 1956; Michaels et al., 1962, Chappaz, 1964; Meingassner et al., 1975) and hamsters (Kradolfer, 1954; Uhlenhuth and Schoenherr, 1955) has

also been investigated with some success. Cavier and Mossion (1956) and other workers found it necessary to ovariectomise the rats and administer estrogen to achieve 'permanent estrous' before infection could be achieved. Indeed, most workers have used exogenous estrogen. These models therefore, while demonstrating successful infections, even if short-lived and not 100% infection rate, present problems of handling if ovariectomy of the animal must be performed before use in the experiment. In addition, the size of the animals means they are costly in terms of maintenance and, if used in chemotherapy studies, drug administration. Hamsters present the additional problem of harbouring natural trichomonads (Uhlenhuth and Schoenherr, 1955) so pre-treatment with drugs and constant monitoring of the infection is necessary to avoid concomitant infections complicating the results.

A murine intravaginal model would be the most suitable for investigation because mice fulfil the requirements of being easy to handle and inexpensive. It is also possible to obtain inbred strains of mouse which should allow more reproducible results. There have been several reports of attempts to develop such a model already (Cappuccinelli et al., 1974; Wildfeuer, 1974; Meingassner, 1977; Landolfo, 1981). The infection rates achieved however, where stated, were generally considerably less than 100% and decreased rapidly with time. However, the longevity of the

infection was not often investigated. All workers agreed that exogenous estrogen is needed to establish the infection, although Cappuccinelli et al(1974) reported that once infection was established the parasites could survive in the vagina in the absence of exogenous estrogen. Wildfeuer (1974) administered corticosteroid to some mice to increase the infection rates but reported that this impaired the state of health of the animals. He also found it necessary to infect the mice under sodium hexobarbitol narcosis. Cappuccinelli et al. (1974) found it necessary to infect the mice on two consecutive days to achieve high infection rates. In no case was the ideal model achieved and, in most of the cases, it appears that the infection rates, longevity and consistency were far from optimal. For this reason the models have been used rather little for studying the host-parasite interaction in trichomoniasis.

1.4. THE VAGINAL ENVIRONMENT

1.4.1. Comparison of the Estrous and Menstrual Cycles

Knowledge of the similarities and differences in the anatomy and physiology of the vagina of humans and mice is necessary to explore murine models of human trichomoniasis. This information could then possibly be exploited to allow modification of the mouse vaginal physiology to mimic that of the human. The morphology and histology of the vagina in both women and mice

changes throughout the menstrual/estrous cycle but these changes cannot be directly compared because the external signs are reflections of different internal events. Until the 1930's confusion was such that menstruation was thought to be a type of estrous. It is thus important to understand the different stages in the estrous and menstrual cycles and their accompanying morphologies if any real comparison between them is to take place.

The patterns of hormonal activity of women have been observed during normal cycles with good agreement between investigators (Abraham et al., 1972; Dhont et al., 1974; Moghissi et al., 1972; Speroff and Vande Wiele 1971). Information on the mouse estrous cycle was reviewed by Bronson et al., (1966). The human menstrual cycle lasts approximately 28 days with the first day of menstruation being number one. Menstruation lasts about 4-5 days and is followed by a follicular or proliferative phase, lasting about two weeks. This phase is similar in the mouse although it is much shorter, and both are characterised by follicle-stimulating hormone (FSH) release from the pituitary to promote follicle growth. Leutinising hormone (LH) then aids the development of the follicle and production of estrogens by the theca-interna cells of the FSH-primed follicle. The rising estrogen levels before ovulation depress the secretion of FSH and trigger an ovulatory

surge of leuteinising hormone with resulting rupture of the follicle and ovulation.

Progesterone is also secreted throughout the preovulatory stage in both the female and the mouse but, in humans, the progesterone is secreted in association with estrogen by the theca-interna cells of the Graafian follicle. In the mouse it is secreted by the interstitial tissue which produces an enormous pre-ovulatory surge of progesterone almost coincident with the LH peak. This forms the essential function of, in combination with estrogen, producing behavioural estrous, marked by increased activity, lordosis and acceptance of the male.

The next stage in both women and mice is that of leuteinisation of the Graafian follicle to form a corpus luteum and secretion of large amounts of progesterone. The fate of the corpus luteum in women and mice then differs. In women the corpus luteum has a life of about 14 days during which it secretes large amounts of 17α-hydroxyprogesterone, progesterone and estradiol 17-β. It then regresses causing a drop in levels of all three steroids and subsequent spasm of endometrial arteries. The epithelium is sloughed off and a new cycle begins.

The murine cycle is such that if no mating occurs during ovulation the corpus luteum never really becomes functional and another estrous cycle starts, making the whole cycle 4-5 days. If mating does occur the

mechanical stimulation of the cervix activates release of prolactin from the pituitary which is needed to render the corpus luteum fully functional. The luteal phase if no pregnancy occurs is then 11-12 days making the whole estrous cycle 13-14 days. This ability to abbreviate the estrous cycle thus increases the chance of successful reproduction by increasing the proportion of time the female will receive the male.

There are thus many differences in the cyclical nature of women and mice, the most obvious being the length of the whole cycle, the times at which the female can receive the male and the relative unimportance of the luteal phase in the unmated mouse. The abbreviation of the luteal phase means that the mouse does not cyclically build up endometrium in preparation for pregnancy. There is thus no regular blood loss. The timing of the cycle in relation to external factors also differs. The period of estrous and timing of ovulation in the mouse are normally controlled by diurnal rhythms of light and darkness. Reversing the time of light and darkness reverses the time of estrous and ovulation (Snell et al., 1940). It is thought that the mid-point of the ovulation is determined more by the mid-point of the dark phase than the onset or completion (Braden, 1957). In contrast, women do not exhibit external influence over their menstrual cycle. A diagram of the human menstrual cycle and the rat estrous cycle found to

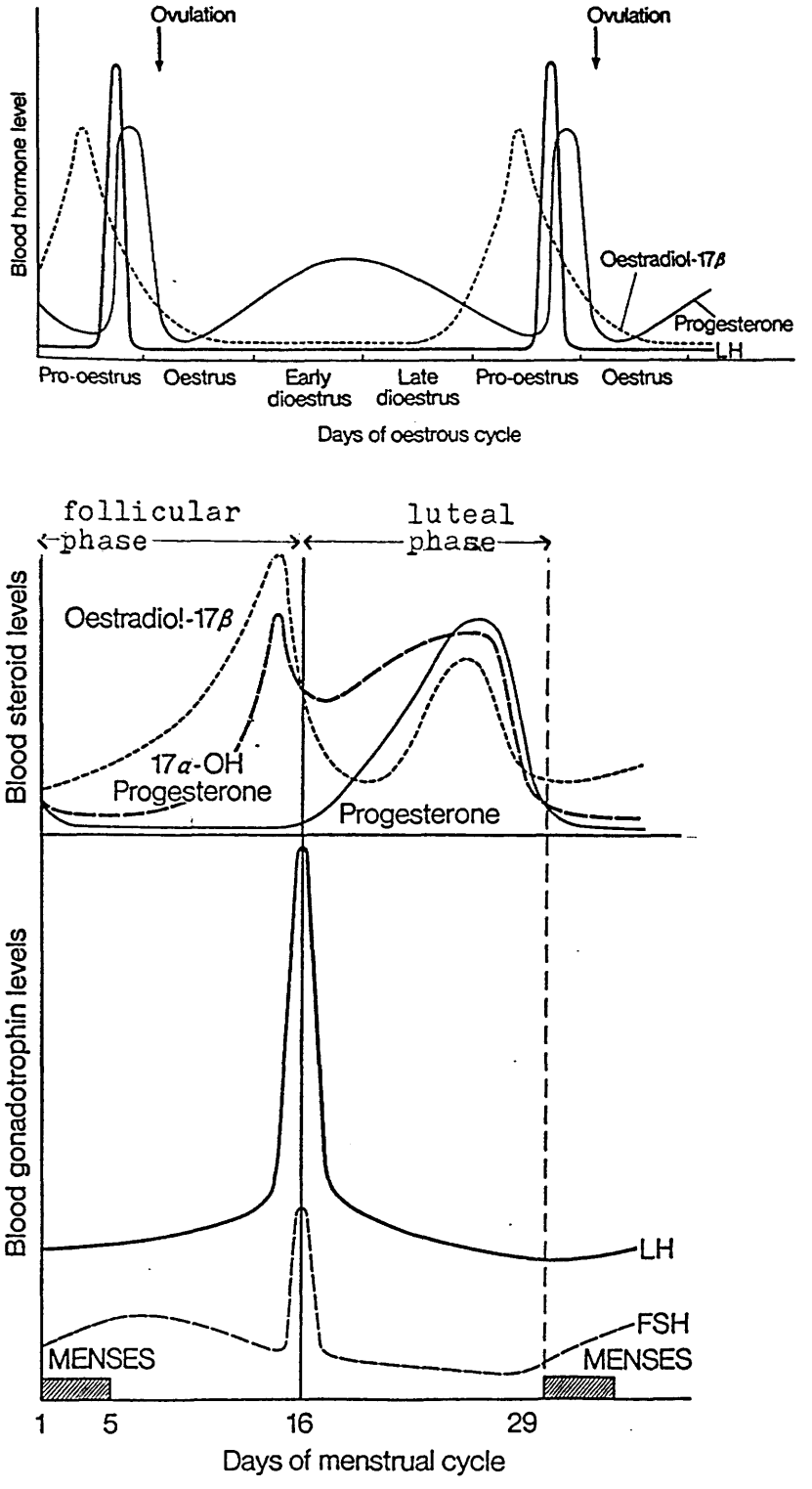
be very similar to the murine cycle) is given in Figure 2.

The changes in hormonal support of the female vagina cause accompanying characteristic changes in the structure. During the follicular phase, when oocytes are undergoing maturation before being ovulated, there is increased mitosis in basal and parabasal layers with an increase in epithelial thickness and number of layers. There is an increased accumulation of glycogen in intermediate transitional and superficial layers and lipid droplets appear during late follicular phase (Averette et al., 1970).

Luteal phase is characterised by desquamation of cells before complete differentiation. The nucleus shrinks, becomes irregular in shape and pyknotic. The edges of the cells roll back giving the characteristic pattern of exfoliation. Exfoliated cells appear wrinkled with loss of, or obscured, surface ridges and are shed into the vaginal lumen. Glycogen is degraded intracellularly by lysosomal enzymes leaving empty spaces (Burgos and Roig de Vargas Linares, 1978).

The effect of the changing hormone level can be shown to have characteristic effects on the murine vaginal tract (Allen, 1922). Vaginal smears taken throughout the cycle faithfully reflect changes in vaginal cytology (Papanicalaou, 1923). This shows that estrogens induce an increased mitotic activity in the

Figure 2. Hormone changes in the rat estrous cycle and the human menstrual cycle



from Short (1972)

vaginal surface epithelium with a tendency to keratinase. The cyclic changes identified thus are given in Table 1 and the accompanying cell types are shown in Figure 3.

In a polyestrous animal, such as the mouse, one cycle is usually followed by another one. The human female enters a prolonged state of anestrous with the vagina and other parts of the genital tract in a regressed state.

The histochemical changes during estrous have also been studied and Balmain et al., (1956) found that considerable amounts of glycogen are found in the mouse vagina after keratinisation and this is probably comparable to the increase in glycogen in the human female during the late follicular phase. Lipid droplets appear possibly slightly later in the cycle in the mouse than in humans.

1.4.2. Composition and Characteristics of Vaginal Secretions

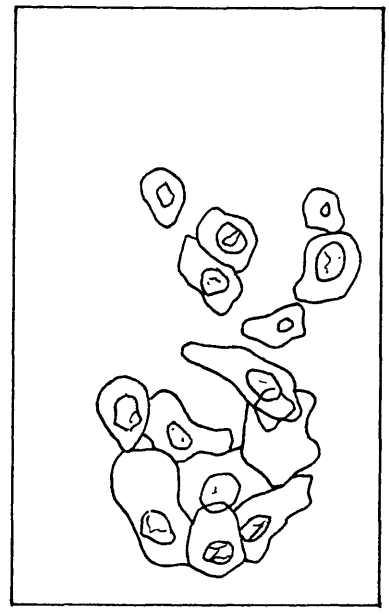
The vaginae of both mice and women are covered with stratified squamous epithelium and contain no glands, except for Bartholin's and Skene's glands near the urethal orifice in women and paired clitoral glands in mice. Vaginal discharge is therefore predominantly from transudate through the vaginal epithelium and from cervical mucus. It contains exfoliating epithelial cells, endometrial and tubal fluid and leucocytes. The

Table 1 Summary of events during estrous cycle of the mouse.

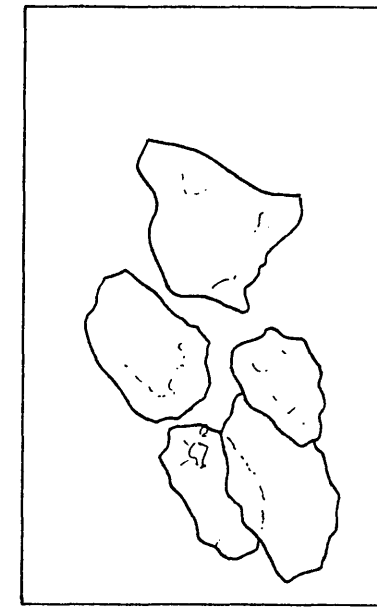
Stage	Length	Vaginal Smear	Associated events
Proestrous	12 hours	nucleated epithelial cells	follicles in ovary growing fast; corpus luteum regresses
Estrous	12 hours	few cornified cells	follicles maximum size; acceptance of male; lordosis
Early Metaestrous	15 hours	many cornified cells	ovulation; cornified layer of vagina becomes detached
Late Metaestrous	6 hours	cornified cells plus many leucocytes	New corpus leteum
Diestrous	57 hours	leucocytes plus nucleated epithelial cells	functional corpus leteum during early part

Adapted from Allen (1922) and Bronson et al. (1966).

Figure 3.

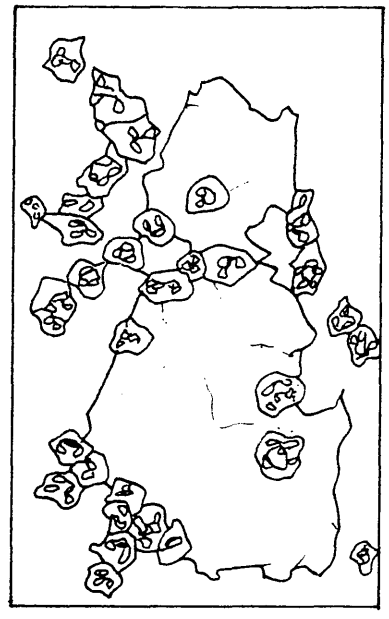


Proestrous

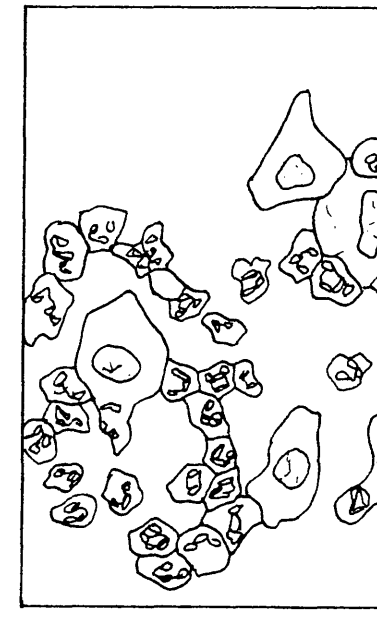


Estrous

Metaestrous



Diestrous



Vaginal cycle in the mouse.
The cell types obtained in a vaginal smear. In proestrous nucleated epithelial cells; in estrous cornified squamous cells only; during metaestrous squamous cells and leucocytes are present, while the diestrous smear is composed of nucleated cells, leucocytes and mucus.

proportion and amount, of each constituent varies throughout the estrous and menstrual cycles. (see Paavonen 1983 for review).

Little information is available on the constituents of mouse vaginal secretions beyond what has already been stated, so most discussion is limited to human secretions. The major organic constituents are proteins, carbohydrates, urea and fatty acid. The vaginal fluid proteins arise from transudation of serum proteins and from proteins produced in the cervix and upper vagina. The major protein components are albumin, immunoglobulins and amino acids (Huggins & Preti, 1981). The vaginal fluid of all women contains acids, primarily acetic and lactic, and in some women they are at high levels. The production normally increases during the mid-cycle and decreases during the luteal phase (Michael, Bonsall & Warner, 1974). Most of these organic acids are thought to arise as metabolic excretions from the vaginal flora which degrade the glycogen in the vaginal epithelium.

The pH of the normal human vagina can vary between 4.1 at day 14 to pH 7.8 at day 2, and depends on the patient and area of vagina. The mean vaginal pH has been found to be significantly higher on day 2, (6.6 $\pm$ 0.3) than day 4 (5.3 $\pm$ 0.3) and day 14 (4.2 $\pm$ 0.2) in all 18 patients investigated (Wagner & Otteson, 1982). Traditionally the pH changes are thought to be due to estrogen causing an increase in the vaginal glycogen content and subsequent degradation of this to lactate by

lactobacilli (Cruickshank and Sharman, 1934). Evidence exists, however, to indicate that estrogen is able to lower vaginal pH before an increase in lactobacillus colonisation (Lewis and Weinstein, 1936; Weinstein and Howard, 1939). The apparent relationship between vaginal acidity and the presence of lactobacilli may simply reflect the fact that relatively few species of bacteria are suited to growth in an acid environment.

The pH of the murine vagina has not been reported but there is information on the vaginal pH of the rat. These two species have similar estrous cycles and so the intravaginal environment may also be similar. It was found that, in complete contrast to the cyclical nature observed in humans, the rat vagina is maintained at around neutral pH throughout the genital tract during the whole estrous cycle (Blandau et al., 1958; Larsen et al., 1976).

1.4.3. The Microbial Flora

The interrelationships between microbial flora and vaginal physiology are not yet understood and literature is sparse in this area. However, it is known that many factors such as glycogen content of epithelial cells, pregnancy, use of contraception, coitus, pH and adherence of bacterial cells do influence the microbial flora. The reproductive hormones produce numerous physiological changes in the host and appears to

influence microbial flora to a large extent (see review Paavonen, 1983).

While presence or absence of ovarian activity has been reported to have no effect on vaginal colonisation in women (Osborne et al., 1974), Larsen et al., (1977) reported that estrogen caused a significant increase in the vaginal flora of rats, particularly noticeable with respect to anaerobes. The influence of normal menstrual cycles on genital microflora has also been documented, although results of different investigations vary considerably. Thus Barlett et. al. (1977) reported that the concentration of anaerobes in the human vagina is maintained at relatively constant levels throughout the menstrual cycle, whereas premenstrually there is a 1000-fold decrease in concentrations of aerobic bacteria. In contrast, Brown (1982) found that aerobic bacteria outnumbered anaerobic in the menstrual and premenstrual cycles and that whereas the number of aerobic bacteria remained relatively constant there was a marked variation with anaerobic bacteria. He reported premenstrual specimens usually contained the highest number of bacteria (approximately 10^9/ml) but the lowest number of species (approximately 11). The menstrual specimens contained the lowest number of bacteria (approximately 4×10^7/ml) and the highest number of species (approximately 14). Most of the difference in counts was due to anaerobic bacteria. Barlett and Polk (1984) found the number of aerobic bacteria to be higher

during the first week of the cycle and subsequently decreased in the premenstrual week. In this case they reported that the number of anaerobic bacteria was relatively consistent at each interval assessed. Larsen and Galask (1982) found a greater number of organisms during menstruation than afterwards. The effect was not selective with respect to anaerobes. Mehta (1982) found that there was no correlation between the hormonal status of the vagina and growth of either aerobic or anaerobic bacteria.

There is little information available on murine bacterial flora but cyclical changes in total numbers have been reported. Corbeil et al., (1985) found a more than fifty-fold increase in bacterial flora at early metaestrous compared to any other stage of the cycle, with there being no bacteria detectable at early proestrous and diestrous. More information is available on the rat vaginal flora which also shows cyclical changes. High numbers of bacteria are present around estrous and low numbers during the remaining part of the cycle (Van der Schoot, 1975; Larsen et al., 1976; Koiter et al., 1977). This information agrees with that reported by Corbiel et al. in finding low numbers of bacteria during diestrous and high bacterial levels are only present if there are low numbers of neutrophils, i.e. around estrous. Larsen et al., (1977) suggested that the cyclical variation in bacterial content of the rat vagina could be explained primarily as the effect of

the secretory pattern of ovarian estrogen. A failure to isolate any anaerobic bacteria from ovariectomised rats has been reported, whereas normal rats were shown to be colonised readily by anaerobic flora (Larsen et al., 1977). They suggested that the estrogen is important in regulating the vaginal flora but because of the time lag between steroid administration and the effect of the hormones on bacterial counts in ovariectomised rats, it is probably mediated rather than direct. Also the finding that plasma estradiol is maximum during proestrous and low during estrous (Smith et al. 1975) whereas bacteria are in highest number during estrous also suggests that a secondary factor is involved.

An association between large amounts of cellular debris in the vaginal lumen of rats and large numbers of bacteria has been reported and it has been suggested that the exfoliated cells are the factor stimulating growth of the bacteria (Koiter et al., 1977). Larsen et al., (1977) report bacterial colonisation occurring co-incidently with cornification. They found the exfoliated cells were colonised by a variety of bacteria and suggested that the effete, exfoliating cells, in the estrogen-stimulated vagina, may release a bacterial growth-promoting substance. The information from Corbeil et al., (1985) also shows many desquamating cells in the murine vagina, with luminal surfaces covered in bacteria. The suggestion of effete cells releasing growth-promoting substances put forward by

Koiter et al. (1977) and Larsen et al. (1977) may thus be relevant to mice as well as rats.

While relative numbers of bacteria are important when comparing rodent and human vaginal flora, it may be just as useful to compare bacterial species colonising the vagina. The flora of the human vagina is controversial but the concept of aerobic lactobacilli as the 'dominant' organism has gradually been modified as the result of quantitative methods and improved anaerobic techniques.

Ideas on the constitution of aerobic vaginal flora have not changed - lactobacilli, corynebacteria, coagulase - negative staphylococci and streptococci are still considered to be the most common organisms (Wilks et al., 1982). What has changed is Döderlein's concept that the vaginal flora is essentially homogenous and consists almost entirely of lactobacilli. Wilks et al., (1982) found variable concentrations of Lactobacillus sp. in patients, it being dominant in two out of five patients. Larsen and Galask (1982) found aerobic Lactobacillus sp. in 87% of patients and Barlett and Polk (1984) and Fair et al., (1970) found aerobic Lactobacillus sp. in 58 and 57 per cent of women, respectively.

The numerically dominant anaerobic bacteria are gram-positive cocci (peptococci and peptostreptococci) and gram-positive bacilli (lactobacilli, eubacteria and

chlostridia) (Barlett and Polk, 1982). However, Hamman (1982) found anaerobic bacteria to be absent from 65% of patients under investigation.

The species of bacteria found in the rat vagina differ considerably from that of humans, reflected by a relatively low presence of lactobacilli (Larsen et al., 1976; Koiter et al., 1977). Larsen et al., (1976) and Yamada et al., (1983) found the dominant organisms to be streptococci and bacteriodaceae. Both papers also report a high frequency of Pasteurella pneumotropica which has not been isolated from the human vagina. Larsen et al., (1976) also noted a high frequency of Proteus sp, which is isolated from less than 10% of human vaginal cultures. The authors speculated the differences in flora may be due to differences in microenvironments provided by humans and rats - especially the difference in pH values. Alternatively there may be differences due to the association of rats with their faecal material in their cages. This, however, is less likely as the predominant organisms in the faeces of rats i.e. lactobacilli, staphylococci, pifidobacteria and clostridia (Mitsuoka & Kaneuchi, 1977) were isolated from the vagina infrequently or not at all (Yamada et al., 1983).

1.4.4. Effect of T. vaginalis on the Vaginal Environment

As mentioned earlier the pH of the normal healthy human vagina is slightly acid (Stamey and Kaufman, 1975;

Parsons et al., 1977; Wagner and Otteson, 1982) and it has been suggested that this is a significant defence mechanism (Stamey and Timothy 1975). In contrast the vaginal pH of women with trichomonal vaginitis is pH 5-6 (Parsons et al., 1977). A change in pH is presumably correlated with a change in bacterial flora. Vaginal fluid from normal women was found to be bactericidal to Escherichia coli, but when the pH was increased with sodium hydroxide, the same fluid supported bacterial growth. Yeaw (1940) found that most bacteria grew well in urine at pH 5-7 but were killed below pH 4.4. It has also been suggested that the bactericidal activity of vaginal fluid may be due to an antimicrobial factor, as was found, but not identified, in prostatic fluid (Stamey et al., 1968).

There is very little information on the association between the normal vaginal flora and trichomonads in humans, but Trussel (1947) and Robinson and Mirchandani (1965) and Thadepalli et al., (1973) found a decrease in lactobacilli in patients with trichomoniasis. Goldacre et al., (1980) reported an increase in the number of anaerobes in patients with trichomoniasis. Parsons et al., (1977) and Pfau and Sacks (1981) found many gram-negative enteric bacteria, mainly Escherichia coli, with a heavier introital colonisation by colonic organisms. In contrast Wilks et al., (1984) found that the presence of T. vaginalis did not greatly affect the vaginal flora when compared to the control group in the study.

1.5 PATHOGENICITY OF *T. VAGINALIS*

The pathogenicity of *T. vaginalis* may depend on many factors such as establishment and colonisation of a suitable site, resistance or evasion of host defence mechanisms, direct tissue damage by the production of toxins or contact-dependant mechanisms, or indirect damage such as inflammation.

Successful colonisation of a surface depends on the adherence of the microorganism to epithelial cells (Smith, 1977) so it is probable that trichomonads must establish themselves rapidly to avoid being washed away with mucosal secretions. Adherence of *T. vaginalis* to epithelial cells has often been reported. Ovčinikov *et al.*, (1975), using specimens from infected patients, reported amoeboid forms of trichomonads with pseudopodia capable of penetrating epithelial cytoplasm. They stated in conclusion that this attachment is for phagocytosis rather than fixation to the epithelium. Krieger *et al.*, (1985), however, ruled out this possibility by the use of radiolabelled target cells. There have been many other reports of attachment of trichomonads to various mammalian cell types (Farris and Honigberg 1970; Heath, 1981; Alderete and Pearlman, 1984; Rasmussen *et al.*, 1986).

The pathogenic effect towards tissue culture has variously been reported to be due to the release of

extracellular toxins or the mechanical process of attachment. There are various reports of vertebrate cell injury due to exposure to cell-free filtrates of old trichomonad cultures (Hogue, 1943; Honigberg and Ewalt, 1961). Nielson and Nielson (1975) also speculated that since trichomonads were not present in every area of epithelial disruption, most cytopathology resulted from cell-free cytotoxins. Pindak et al., (1986) supported this view. Although they confirmed that a focal lesion is produced by adding T. vaginalis to a culture of mammalian cells, they observed that the lesion expanded until the entire monolayer was destroyed and a substance in the medium prevented the attachment of a new monolayer.

The other possible mechanism of pathogenesis is that some mechanical process is involved that is independant on the attachment of the trichomonad to the target cell. A report on the haemolytic activity of trichomonads supports this proposition (Krieger et al., 1983). Alderete and Perlman (1984) concluded that the release of a toxin responsible for cell destruction is unlikely because they observed the effect of the trichomonad was always focal, with some apparently normal cells always present, and cell-free supernatants from cytopathogenic T. vaginalis had no effect on mammalian cells.

1.6 HOST IMMUNITY TO *T. VAGINALIS*

Infection with *T. vaginalis* may cause inflammation of the vagina and cervix with subepithelial infiltration by plasma cells, lymphocytes and neutrophils (Nielson & Nielson, 1975). However, as asymptomatic infections are common it follows that in many cases the host response is minimal, possibly due to parasite evasion of the immune response. The findings that some infections are long-lived and that reinfection can occur implies that no protective long-term immunity exists.

There is reasonable evidence that the female genital tract, particularly the cervix, is able to build up a local exocrine response. Infection of the genital tract with various microorganisms has been shown to induce local antibody production; for example uncomplicated gonorrhoea (O'Rielly *et al.*, 1976), *Candida albicans* (Waldman *et al.*, 1972) and inactivated polio virus (Ogra & Ogra, 1973). Trichomoniasis itself induces a marked rise in the number of immunoglobulin-bearing plasma cells following infection (Chipperfield & Evans, 1972). Ackers *et al.*, (1975) found immunoglobulin A (IgA) versus *T. vaginalis* in vaginal secretions from 76% of women with trichomoniasis compared with 42% of uninfected women. The secretory IgA titres were twice as high in infected women compared with uninfected women. Su (1982) detected IgG versus *T. vaginalis* in 70.8% of secretions from infected women

compared to 23.3% of non-infected women. Of the 17 antiparasite secretions, only 2 were of IgA. No correlation could be observed between the severity of the disease and the antibody level in the patient.

The presence of serum antibodies versus T. vaginalis is just as confusing as they are commonly found in patients who have had no contact with the parasite. Some investigators (Teras, 1961; Nigeson, 1966) consider titres of 1:80 to 1:160 as non-specific when evaluating agglutination tests and most investigators agree that there is not a single, reliable diagnostic test for trichomoniasis involving the immune response.

The origin of the antibody in the cervico-vaginal secretions of infected patients is also unknown and it must be remembered that the finding of antibody at a secretory site does not necessarily indicate that the antigen stimulated the immune response at that site. This is especially important in trichomoniasis as common antigens have been reported between the vaginal and intestinal trichomonads (Kott & Adler 1961; Su, 1982). The low levels of IgA compared to IgG found in the secretions by Su, (1982) may indicate that it is not a local secretory immune response that is proceeding (Vaerman & Ferin, 1977). The importance of antibodies in both the genital tract and the serum is not known but because of the non-invasive nature of trichomoniasis the usefulness of serum antibodies is doubtful.

The role of non-specific immune responses also remains unknown in trichomonad infections but the potential importance of neutrophils is clear as they are present in high numbers during at least part of the menstrual cycle. Clusters of leukocytes have been described as "the single most valuable indication of the presence of the organism" i.e. T. vaginalis (Frost, 1962). Viable T. vaginalis releases factors which are leucocytotoxic in vitro (Mason and Forman, 1980) and polymorphonuclearleucocytes have been shown to possess trichomonicidal activity via reactive oxygen intermediates. The reaction is independent of antibody but may involve complement (Rein et al., 1980). Other authors report activation of complement by the alternative pathway (Holdbrook et al., 1982) which may be important because of a release of factors leading to an influx of neutrophils. Martinotti et al. (1983) reported macrophage cytotoxicity versus trichomonads, but the in vivo importance is not known.

1.7. AIMS OF THE PROJECT

The main purpose of this project was to develop a murine model of disease suitable for use in studies of intravaginal trichomoniasis. It was hoped that it would be reliable and the infection sufficiently tenacious and long-lasting to allow further study of the host-parasite relationship. To this end several approaches were followed. Firstly, the study of the influence of host physiology on the course of disease, such as the use of

experimentally-manipulated mice and exploitation of important genetic variation. Secondly, a study of the vaginal micro environment with the aim of determining factors important in establishing disease and using this knowledge to modify the environment in ways favourable towards the establishment of infection. Thirdly, to evaluate the influence of characteristics of the parasite isolate on the establishment of an infection.

It was hoped to use the model developed to elucidate the interactions between the parasite and the host, particularly to evaluate the immune response to infection and to attempt to understand the reasons for the location of the parasite in the vagina.

MATERIALS AND METHODS

2.1 MICE

Female mice were purchased 3-5 weeks old from Bantin and Kingman (Hull, England) or bred in the Department of Zoology, Glasgow University. Mice were matched for age and source, as far as possible in all experiments.

2.2. PARASITES

Three lines of Trichomonas vaginalis, named 6950♂, 39 and G3 were used throughout this study. Line 6950♂ was originally isolated from a symptomatic male and obtained from Dr J. Ackers (London School of Tropical Medicine and Hygiene). Line 39 was originally isolated from a patient at the genito-urinary clinic at Glasgow Royal Informary and line G3 is a cloned line from Bushby's strain which had been maintained axenically in vitro for more than 20 years. Maintenance of the parasites was in modified Diamonds Medium (MDM) (Diamond, 1957) supplemented with 10% heat-inactivated horse serum (HIHS) containing benzyl penicillin (1000 U/ml) and Streptomycin (1 mg/ml). Incubation was at 37^{o}C and the lines were subpassaged every two to three days.

2.3. CRYOPRESERVATION OF T. VAGINALIS

A 10% (v/v) solution of sterile dimethylsuphoxide in MDM with 10% (v/v) HIHS and penicillin and

streptomycin was prepared. To this was added an equal volume of an axenic log-phase culture of *T. vaginalis*. The solutions were mixed and aliquoted into Nunc 2 ml cryotubes. The tubes were placed in a polystyrene container and floated overnight on liquid nitrogen before being placed in the liquid nitrogen.

2.4. INFECTION OF MICE WITH *T. VAGINALIS*

The standard inoculation protocol was as follows: two days before exposure to parasites Balb/c mice aged 10-12 weeks were administered estradiol - 17β -cypionate (Sigma 5 mg/ml, prepared by sonication in a MSE Soniprep 150, using an exponential microprobe, and 4 25-second bursts of 6 μm amplitude sonication, with a 20-second cooling period between bursts). Estrogen was administered subcutaneously except where stated.

Trichomonads were prepared by centrifugation (1000 g, 10 minutes) of a log-phase culture (40-150 x 10^4/ml) and resuspended in MDM plus 0.3% (w/v) bacto-agar (Difco), but no antibacterials or serum, to 5 x 10^6/ml. 10^5 trichomonads were inoculated directly into the vagina using a round-ended needle. The vagina was plugged with a small, dry sterispon plug (Allen and Hayburys, England) where indicated.

Ten to eighteen days after parasite inoculation the vagina was washed out with 0.2 ml MDM. These 'washouts' were added to 1 ml of the MDM with 10% HIHS and

penicillin, streptomycin and nystatin (20 U/ml) and placed in a Linbro 24-multiwell plate (Gibco). This was placed in an anaerobic incubator (Whitley Anaerobic Cabinet MarK II) and examined daily for motile trichomonads for up to 7 days using an inverted microscope. A culture arising from a 10 day post-infection washout was inoculated into fresh medium a day or two before use for infection of more mice.

2.5. METRONIDAZOLE-TREATMENT OF MICE

Metronidazole (May and Baker Ltd, England) was formulated in 0.2% cellacol in distilled water and ball-milled overnight. It was administered orally at a dose of 50 mg/kg body weight (0.1 ml/mouse) daily on days 3 to 8 post-infection.

2.6. OVARIECTOMY OF MICE

Mice were sedated using Sagital (May and Baker, England) (20% solution in 20% ethanol, 0.1 ml/10 g body weight). A bilateral ovariectomy was performed via a dorsal incision. The mice were allowed to recover for at least 3 weeks before use in an experiment.

2.7. IRON-TREATMENT OF MICE

Ferric ammonium citrate (Sigma, England) was dissolved in phosphate buffered saline (0.02M Na_2HPO_4, 0.02M NaH_2PO_4 pH 7.4) at a concentration of 20 mg/ml.

This was administered immediately, 0.1 ml per mouse intraperitoneally. Mice were inoculated with parasites one day later.

2.8. VACCINATION OF MICE

2.8.1. Preparation and use of SolcoTrichovac

SolcoTrichovac was prepared, according to the manufacturer's instructions, by reconstitution of the repolymerized gelatine (containing lactobacilli, gelatine and phenol) with the sterile diluent provided. It was then used immediately.

The protocol was as follows: mice were either injected intraperitoneally with the full human dose or intramuscularly with 2/5 of the human dosage. This was repeated twice at 10 day intervals. Five days after the third administration of SolcoTrichovac the mice were estrogen-treated. Infection was 2 days later, following the standard procedure.

2.8.2. Preparation of trichomonad lysates for use as vaccine

Trichomonads, from a log-phase culture, were prepared by being washed twice with PBS, pH 7.4; and then resuspended in PBS to a concentration of 0.5 - 2.5 x 10^6/ml. They were then either sonicated while cooled on ice (using an MSE Soniprep 150 fitted with an

exponential microprobe and programmed to deliver 4, 10-second bursts of 6 μm amplititude with 10-second cooling periods between bursts] or subjected to cycles of freezing (-20^{o}C) and thawing (37^{o}C) until viable trichomonads were not visible microscopically.

The vaccines in Freund's complete adjuvant (FCA) were prepared by mixing equal volumes of FCA (Sigma, England) and the trichomonad lysate until a homogenous emulsion was obtained. Other vaccines were diluted with PBS only.

2.8.3. Vaccination Procedure

The mice were vaccinated with lysates of 1-3 x 10^{6} trichomonads, 0.1 ml per mouse. The 'vaccines' prepared with FCA were administered subcutaneously and the other 'vaccines' intraperitoneally or subcutaneously as indicated. This was repeated twice at 10-day intervals. Estrogen was administered 3 days after the last injection and the standard infection protocol followed 2 days later.

The oral vaccine was prepared by freezing and thawing (as described in section 2.8.2). 0.1 ml of the suspension, adjusted to contain the appropriate amount of protein, was administered by the use of a round-ended needle.

2.9. DETERMINATION OF ESTROUS

The state of estrous was determined by gently scraping the vaginal wall with a round-edged instrument and mixing the vaginal material so obtained with a drop of PBS, pH 7.4, on a slide. Examination of the slide was then performed after fixing with methanol and staining with Giemsa stain (Gurr, England).

2.10. DETERMINATION OF THE pH OF VAGINAL WASHOUTS

Vaginal washouts were performed using a syringe and a round-ended needle. 0.03 ml of water, measured as accurately as possible using the syringe, were washed into the vagina and the needle gently rubbed around the sides of the vagina. The resulting suspension was then removed and each washout placed individually in an Eppendorf vial. The tubes were left standing for approximately one hour to allow large particles to settle and then the pH was measured on a Radiometer BMS 2, MK 3 Blood Micro System pH meter. The pH of each washout was measured twice or, if there was enough supernatant, three times.

2.11. ASSESSMENT OF ACTIVITY OF ZINC AND IRON AGAINST *T. VAGINALIS IN VITRO*

Modified Diamond's Medium, supplemented with 10% HIHS, penicillin and streptomycin, and with the appropriate concentration of zinc chloride or iron

sulphate were prepared and 19 mls of each medium were aliquoted into sterile universals. To this was added 1 ml of an axenic log-phase culture of T. vaginalis of known density. The initial density of T. vaginalis was then calculated and the cultures incubated at 37°C. At appropriate times, samples were removed, using a sterile pipette, and the parasite density in each culture determined using an improved Neubauer haemocytometer. Each experiment was performed in duplicate.

RESULTS

3.1. THE MURINE MODEL OF TRICHOMONIASIS

3.1.1. Typical infection rates of Balb/c mice inoculated intravaginally with T. vaginalis 6950♂ and 39

Mice were intravaginally inoculated with *T. vaginalis* following the standard protocol, and ten days later the mice were examined for the presence of infection. Any trichomonads present in the vaginal washouts were maintained *in vitro* for four days before use in infection of further mice. The whole procedure therefore took fourteen days. As infection of mice was usually performed weekly it was necessary to simultaneously maintain two passage lines of both 6950 ♂ and 39. Fig. 4 shows the results of these weekly experiments over a period of more than a year.

Infections were usually obtained with both lines but the infection rate was variable, however, ranging from 0 to 100% with infection rates being 50% or over in more than two-thirds of the experiments. The variability occurred with both 39 and 6950 ♂ indicating that it was not a property of just one line of trichomonad. The reason for the differences in susceptibility to infection with *T. vaginalis* between experiments was not clear but it was a drawback of the model. Therefore experiments were designed with the aim of increasing the infection rate obtained to be consistently 100% and also discovering the reason for the variation.

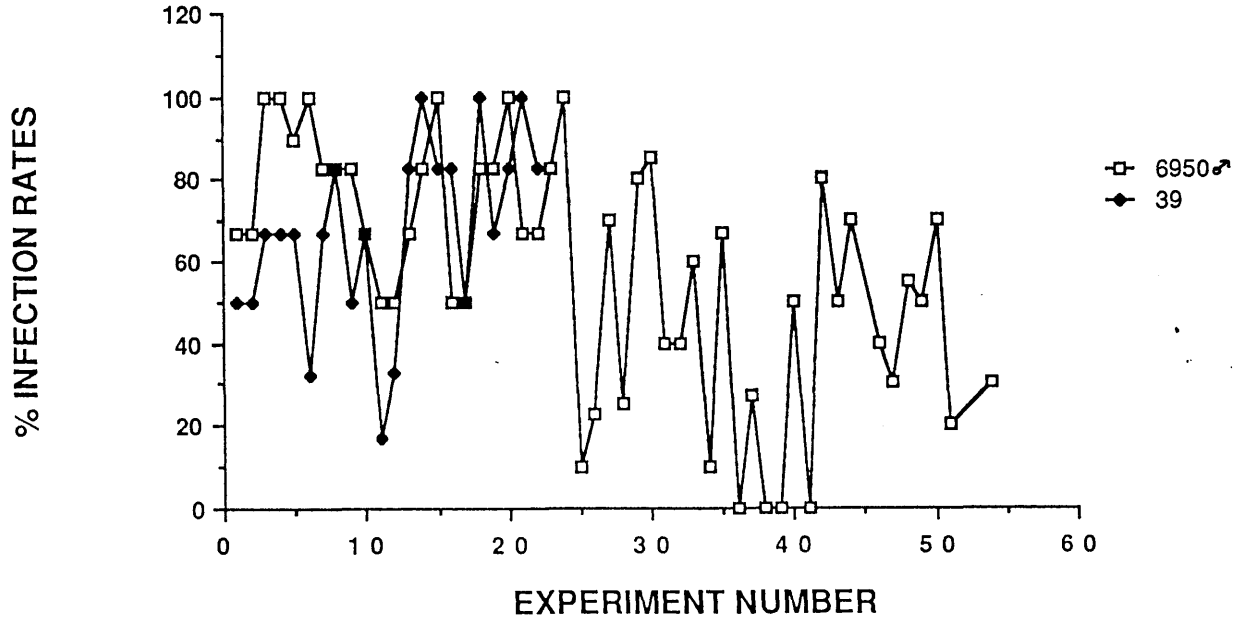


FIGURE 4 INFECTION RATES OF T. VAGINALIS INTRAVAGINALLY INOCULATED INTO BALB/C MICE OVER TIME.

3.1.2. The variation of infection rates within experiments

The extent of variation in the infection rates that can occur within a single experiment was determined by separating a large group of inbred Balb/c mice of the same age, not all siblings but randomised, into separate cages and then inoculating them with *T. vaginalis* using the standard infection procedure. Table 2 details the results. Overall they show that there was some variation between groups but less than that between experiments. The infection rates within an experiment tended to be either consistently high or consistently low - experiment 1, group 4 being an exception to this rule.

As the infection rates within an experiment were fairly consistent, this model could be successfully employed in experiments comparing infection protocols and was also suitable for use in chemotherapy experiments. It was not ideal for the latter purpose, however, the variability meaning that one experiment would not be sufficient to indicate that a drug has no therapeutic value.

3.2. THE INFLUENCE OF HOST PHYSIOLOGY ON SUSCEPTIBILITY TO INTRAVAGINAL INFECTION BY *T.VAGINALIS*

3.2.1. The use of experimentally-manipulated mice

3.2.1.1. The effect of estrogen treatment

The effect of estrogen treatment of mice prior to inoculation of parasites was determined by comparing

Table 2 The extent of variation within an experiment involving intravaginal infection with *T. vaginalis* of identically treated mice.

Group number	Number of mice infected/group size at 10 days Experiment Number				
	1	2	3	4	5
1	4/6	6/6	5/6	5/6	2/6
2	4/6	5/6	5/6	6/6	3/6
3	5/6	5/6	6/6	6/6	1/6
4	2/6	4/6	3/6	3/6	-
5	4/6	4/6	3/6	-	-
6	5/6	-	-	-	-

- not done

a all mice were infected using the standard protocol and *T. vaginalis* 6950 ♂

infection rates of estrogen-treated mice and mice that had been similarly inoculated with corn oil as a control. The results are given in Table 3a. They show that while infections were not established in the control mice, such that parasites were not recovered at 10 days post-infection, more than half of the experimental group were infected at that time. These results clearly indicate a requirement for estrogen treatment of mice.

The route of administration of estrogen was found to be relatively unimportant as similar infection rates were obtained if the mice were injected subcutaneously or intraperitoneally or the dose split equally between these routes. The results of these experiments are detailed in Table 3b.

The importance of the timing of the treatment was also evaluated. This was performed by administering estrogen at various times before inoculation of the parasites from four days to a few hours. The results of the experiments are given in Tables 3c and 3d. They show that estrogen must be given more than a few hours before inoculation of the parasites; but that apart from this limitation the timing does not appear to be crucial. Extending the period to four days, however, resulted in slightly fewer mice becoming infected.

An experiment to determine the relationship between route and timing of administration of estrogen was

Table 3 The effect of estrogen on intravaginal infection of mice with *T. vaginalis*

Variable procedure	route/time	Number of mice infected / group size	
		days post-infection	
		10	25
a *Requirement for estrogen treatment of mice*			
No estrogen treatment		0/34	-
40 mg/Kg estradiol cypionate		23/40	-
(pooled results from four experiments)			
b *Route of estrogen administration*			
	ip sc	4/6	2/6
	sc	4/6	4/6
	ip	5/6	3/6
c *Timing of estrogen administration*			
2 hours	before infection	0/6	-
25 "	" "	29/48	-
30 "	" "	2/6	-
2 days	" "	30/48	-
3 "	" "	14/24	-
4 "	" "	4/12	-

d *The effect of varying the route and timing of administration of estrogen*

	Days between estrogen administration and inoculation of parasites		
	1	2	3
Route sc	6/8	7/8	7/8
sc + ip	7/8	7/8	7/8

a. Standard protocol used in all cases except for parameter under investigation.

b. Inoculation intravaginally of 5×10^6 trichomonads in MDM with 0.3% agar.

\- Not done

performed by administering estrogen to the mice by subcutaneous or intraperitoneal routes either one, two or three days before inoculation of parasites. This showed that, within the chosen parameters, the infection rates do not depend on routes or timing of estrogen administration (Table 3e).

These experiments demonstrated that estrogen treatment of the mice before inoculation of the parasites was a necessity if infections were to be established.

The dose of estrogen required was studied and the results are given in Table 4. It was found that the dosage that is administered could be varied considerably without affecting the infection rates. A dose as low as 0.5 mg estradiol cypioniate per kg body weight was effective. Four experiments were performed (Table 4) using various doses and within each experiment there were similar infections rates for all the dosage levels used.

3.2.1.2. Effect of bilateral ovariectomy

The effect of removing endogenous estrogen by bilateral ovariectomy of the mice a few weeks before experiments commenced and subsequently treating with exogenous estrogen two days before infection was also determined. The results from two experiments are given in Table 5. In neither case was it found that ovariectomy resulted in increased infection rates and in

Table 4 The effect of estrogen at different dose levels[a]

estrogen (mg/kg body weight)	Number of mice/group size infected on day 10
Experiment 1	
40	5/6
30	5/6
20	6/6
10	6/6
Experiment 2	
40	3/6
30	2/6
20	2/6
10	4/6
Experiment 3	
40	3/10
10	3/10
6	5/10
3	4/10
0	0/10
Experiment 4	
40	7/10
4	6/10
1	8/10
0.5	7/10
0	0/1

a. estrogen administered as a single subcutaneous dose two days before parasite inoculation unless stated otherwise.

b. estrogen given 1 day before inoculation.

Table 5 The effect of bilateral overiectomy of mice on their susceptibility to intravaginal infection with *T. vaginalis*

Procedure	Number of mice/group size infected on day 10	
	Experiment	
	1	2
ovariectomised mice[ac]	2/5	1/4
intact mice[bc]	6/12	5/6

a the ovariectomy was performed 3-4 weeks before the mice were used in the experiment.

b intact mice were not subjected to a sham operation.

c both groups of mice were subjected to estrogen administration before inoculation of *T. vaginalis* line 39 following the standard procedure.

experiment two the infection rate of the ovariectomised animals was markedly lower than in the control group. It should be noted that the control group were not subjected to sham operations.

3.2.1.3. Effect of diet fed to mice

An investigation of the effects of the diet of the mice, on susceptibility to T. vaginalis, was performed by weaning groups of mice onto different diets - 41B or CRM. The results of the experiment are given in Table 6. Those of experiment 1 suggested that diet could be an important factor. The findings were not confirmed in experiments two and three, however, and overall the results indicate that the diet given to the mice is not by itself the factor determining their susceptibility, to infection consequently all mice were subsequently fed with diet 41B.

3.2.1.4. The effect of iron-treatment of mice prior to inoculation of parasites

The effect of pre-treatment of the mice with ferric ammonium citrate was also assessed. Groups of control mice were administered the same volume of PBS. The results of eight experiments are detailed in Table 7. In each of the experiments the infection rates for iron-treated mice were higher than those for PBS-treated mice. When the results were expressed as a percentage,

Table 6 The effect of their diet on the susceptibility of mice to *T. vaginalis*[a]

Diet	Number of mice/group size infected on day 10		
	Experiment		
	1	2	3
CRM	8/10	5/10	3/6
41B	2/6	7/10	3/6

a mice infected with *T. vaginalis* 6950 ♂ using the standard protocol.

Table 7 The effect of iron treatment on the susceptibility of mice to intravaginal infection with Trichomonas vaginalis

Experiment number	Number of mice infected at day 10/group size iron treated[a]	control[b]
1	8/9	4/10
2	4/10	3/10
3	6/6	7/7
4	1/6	0/6
5	6/10	4/10
6	9/10	8/10
7	4/10	3/10
8	5/10	2/10
mean infection rates % ± SD	60.8 ± 29.5	35.4 ± 22.8

The infection rates were shown to be significantly different ($P < 5$) using the paired t-test.

a ferric ammonium citrate was administered intraperitoneally 100 mg/kg body weight, in 0.1 ml PBS, 1 day before inoculation of the parasites, line 6950♂.

b 0.1 ml PBS was administered intraperitoneally as a control.

so that results for each group could be compared, the difference between the groups was found to be significant. The results therefore suggest that pre-treatment of the mice with iron increases their susceptibility to *T. vaginalis* inoculated intravaginally.

3.2.1.5. The effect of testosterone-treatment of mice

It has been reported that single injections of testosterone propionate to five day old mice resulted in constant estrogen secretion and persistantly cornified vaginal mucosa (Barraclough, 1961). An experiment was designed to determine the effect of this treatment on the susceptibility of mice to intravaginal infection with *T. vaginalis*.

Mice were injected with testosterone (1mg per mouse, in cornoil, subcutaneously) at 5 days old and then left for 10 weeks before further experimentation. They were then administered corn oil subcutaneously (0.2 ml), as a control, following the standard protocol. Two days later they were vaginally examined for the stage of estrous and inoculated intravaginally with *T. vaginalis* 39. A control group was set up by treating them with estradiol, using the standard procedure described in the materials and methods section, and then vaginally examining and inoculating them with *T. vaginalis* in an identical fashion to the other group.

All mice, in both groups, were found to be in estrous at the time of inoculation of parasites. The results concerning susceptibility (Table 8) are conflicting. In experiment 1 testosterone-treated mice were found to be more susceptible to infection than control mice. In contrast the results of experiments 2 and 3 indicate that testosterone-treatment alone does not increase susceptibility to infection. It is noteworthy that by day 10 post-infection most mice of the testosterone-treated groups that became infected in both experiments had resolved their infections.

Despite the somewhat contradictory results of the different experiments, it is intriguing to note that some testosterone-treated mice, although they had received no estradiol prior to infection, were susceptible to infection for at least five days. It would be interesting to perform this experiment again with the addition of a control group that had neither been testosterone-treated nor received any estradiol and in which all the control groups were sham injected at 5 days old.

Testosterone-treated mice, subsequent to resolving their infections, were again vaginally examined to discover their status with regard to estrous. These examinations revealed that permanent estrous had not been achieved by testosterone administration.

Table 8 The effect of testosterone-treatment of mice on their susceptibility to infection with T vaginalis 39

	Treatment	Number of mice infected/ group size	
		5 days	10 days
Experiment 1	Testosterone[a]	6/6	1/6
	Control[b]	4/6	2/6
Experiment 2	Testosterone[a]	0/6	1/6
	Control[b]	5/6	5/6
Experiment 3	Testosterone[a]	0/6	0/6
	Control[b]	6/6	6/6

a testosterone was administered to 5 day old mice. The mice were injected with cornoil and inoculated with T. vaginalis when 11 weeks old.

b control mice were estradiol injected and inoculated intravaginally with T. vaginalis following the standard protocol at 11 weeks old.

3.2.2. The influence of genetically-based variations upon mouse susceptibility to intravaginal infections with T. vaginalis

3.2.2.1. The effect of age

The influence of age on the infection rate was determined by inoculating parasites into mice of between eight weeks and a year in age. The results (given in Table 9) indicate that age of mouse alone is not crucial in determining whether trichomonads colonise the vagina.

3.2.2.2. The susceptibility of different mouse strains

The influence of the genetic background of the mouse was evaluated by attempting to infect various strains of mice using the standard protocol. The results are presented in Table 10. They show that all the strains of mice investigated could be infected. There was, however, considerable variation in infection rates at 10 days, both between groups of mice of the same strain and mice of different strains. C3H/He mice were relatively refractory to infection and only one out of the thirty mice used was infected at 10 days. The highest infection rates were obtained with DBA/2, CFLP, CBA and Balb/c mice that had been bred in the department.

Table 9 The effect of age of mouse on infection rates

Experiment	Age of mouse (months)	Number of mice/group size infected at 10 days	18 days
1[a]	2	2/5	
	7	2/5	
2[b]	3.5	10/12	
	11	4/5	
3[b]	4	3/6	
	9	4/6	
4[b]	2.5		4/6
	6		0/6
	9		3/6
	12		3/6
5[b]	2.5	7/20	
	6	5/10	

a mice intravaginally inoculated with T. vaginalis, line 39.

b mice intravaginally inoculated with T. vaginalis, line 6950♂

Table 10 The susceptibility of different strains of mice to intravaginal infection with *T. vaginalis*

Mouse strain	Number of mice/group size infected at 10 days				
	Experiment				
	1	2	3	4	5
Balb/c (M3[a])	6/8	7/9	-	2/10	-
Balb/c (B+K)	1/8	0/9	4/10	5/10	1/10
DBA/2	5/10	4/6	4/10	-	-
C3H/He	0/10	1/10	0/11	-	-
C57/Bl	2/10	2/8	1/8	-	-
CFLP	-	-	-	6/6	3/5
CBA	-	-	-	4/10	7/10

a one group of mice (M3) were bred in the Department of Zoology, Glasgow University. The other group (B&K) were purchased from Bantim & Kingman.

- not done.

3.2.2.3. The variation in susceptibility of groups of mice

Variation in susceptibility of mice within a group was a constant feature and an experiment was designed to determine if susceptibility was a permanent or temporary characteristic. Six groups of mice of different ages were inoculated with parasites and ten days later the infection rates were determined. A month later the procedure was repeated using some mice from the same groups as those used in the first experiment and others of similar ages. The two halves of the experiments were identical as far as possible but there was a difference in the trichomonads used. The trichomonads used in the first half of the experiment was a 6950 ♂ culture that had been in stabilate until a week before use in the experiment. The trichomonads used in the second half of the experiment came from the same original 6950 ♂ culture but had been subpassaged intravaginally in mice using the standard protocol for more than a year.

The findings are detailed in Table 11. There was considerable variation in infection rates in both halves of the experiments. Most relevant, however, was the finding that mice from that were completely resistant to infection during the first part of the experiment became infected during the second part of the experiment suggesting that, at least in some cases, resistance to infection was a temporary rather than a permanent feature or related to the properties of the parasite rather than the mice.

Table 11 The effect of time of arrival and time of infection on the course of the disease.

Mouse batch	Number of mice infected at 10 days/group size	
Date of arrival	Date of inoculation of parasites 14.4.86[a]	9.5.86[b]
14.11.85	-	3/6
4.12.85	5/6	-
16. 1.86	0/6	5/6
13. 2.86	2/6	-
18. 2.86	-	6/10
27. 2.86	0/6	-
13. 3.86	0/6	3/6
27. 3.86	-	2/6
23. 4.86	-	1/6

a 6950 ♂ trichomonad culture maintained in stabilate until one week before use in experiment.

b trichomonads that had been subpassaged intravaginally in mice using the standard protocol for more than a year.

3.3. CHARACTERISTICS OF THE PARASITE ISOLATE THAT AFFECT ITS ABILITY TO INFECT MICE INTRAVAGINALLY

3.3.1. Comparison of the infectivity of lines 6950 ♂ and 39

An experiment was performed to compare the infectivity of lines 6950 ♂ and 39 during long term subpassaging between mice. The cultures were maintained identically throughout the experiment using the standard protocol described in the Materials and Methods section. The vaginal infection of mice with lines 39 and 6950 ♂ was carried out over 23 passages from mouse, via *in vitro* culture, to mouse and the infection rate at 10 days determined for each passage. The results are given in Table 12. The infection rates were expressed as a percentage and the averages for both lines calculated. Statistical analysis using the paired t-test showed that there is no significant difference between the infection rates with lines 6950 ♂ and 39, although there was a greater variation between passages with line 39. Note that all mice were found to be infected on five occasions with line 6950 ♂, but only on three occasions with line 39.

3.3.2. The effect of *in vitro* maintenance on parasite infectivity

The effect, on the infectivity of the parasites, of maintaining them axenically *in vitro* continuously or

passaging them intravaginally in mice, using the standard protocol was also evaluated. The results from two experiments show that up to three months axenic *in vitro* cultivation had very little effect on the infectivity of the parasite. Conversely, subpassage through mice did not result in enhanced infectivity of the parasites. However, comparison of the infectivity of the three lines of *T. vaginalis*, 39, 6950 ♂ and G3, show that there were considerable differences between them (Table 13). No intravaginal infections of mice with line G3 were observed, whereas at least 50% of the mice inoculated with other trichomonads were infected at 10 days. Line G3 is a clone of *T. vaginalis* line originally isolated more than ten years ago and maintained axenically *in vitro* by serial subpassage since then. In contrast lines 6950 ♂ and 39 had been isolated from patients in recent years and maintained in liquid nitrogen since then, except for a period up to a year before experimentation when they had either been serially subpassaged through mice using the standard procedure or maintained axenically *in vitro*

3.3.3. The effect of the growth phase of the trichomonad in vitro

The importance of other parasite features, such as the growth phase of the parasite, when harvested before use for inoculation of mice, to infectivity were also evaluated. Parasites at different phases of growth *in vitro* were obtained by seeding cultures with a range of

Table 12 A comparison of the infection rates with *T. vaginalis* lines 6950 ♂ and 39.

Week of experiment number	Number of mice infected at day 10/group size	
	T. vaginalis line	
	6950 ♂	39
1	4/6	3/6
2	4/6	3/6
3	6/6*	5/6*
4	6/6	4/6
5	12/12	11/12
6	9/10	4/6
7	6/6	5/6
8	5/6	4/6
9	5/6	5/6
10	5/6	5/6
11	4/6	1/6
12	3/6	2/6
13	3/6	5/6
14	4/6	6/6
15	5/6	5/6
16	6/6	5/6
17	3/6	6/6
18	3/6	3/6
19	5/6	6/6
20	5/6	4/6
21	6/6	5/6
22	4/6	6/6
23	4/6	5/6
mean % infection ± SD	79.2% ± 15.32	68.6% ± 26.4

No significant difference between the groups at 5% level.

* infection rates are for all vaginal washouts on day 10 post-infection except where marked * were the results for day 5 washouts are given.

Table 13 The effect of a period of *in vitro* maintenance of *T. vaginalis* on its infectivity to mice intravaginally[a]

Experiment	passaged intravaginally in mice: Number of passages	Length of time (months)	Length of axenic cultivation	Line	number of mice infected/group day 10	group size
1	26	13		6950 ♂	7/10	
			1 week[b]	6950 ♂	9/10	
			3 months[b]	6950 ♂	7/10	
			> 10 years	G3	0/10	
2	21	12		39	6/6	
	19	12	1 month	39	5/6	
	23	12		6950 ♂	5/6	
			1 week[b]	6950 ♂	6/6	
			> 10 years	G3	0/6	

[a] using the standard protocol involving an *in vitro* culture step as described in the materials and methods.

[b] these cultures were from the same original culture of 6950 ♂ but had been maintained in liquid nitrogen until the stated period before use in the experiment.

numbers of trichomonads so that they could be harvested on the same day for inoculation into mice. Thus all the cultures for the experiment were from a single source and had been cultivated *in vitro* for the same time. The results obtained for this experiment are given in Table 14. They show cultures in log phase resulted in higher infection rates. No infections were produced by trichomonads from lag phase.

3.3.4. The number of parasites required to produce an intravaginal infection

The effect of altering the number of trichomonads inoculated was also determined. The results are detailed in Table 15. They show that infections were produced even when the number of parasites inoculated was reduced 100-fold whereas increasing the dose 10-fold did not ensure 100% infections. Clearly the number of parasites inoculated is not the major factor in determining susceptibility of mice to infections.

3.3.5. The effect of maintenance of T. vaginalis in various mammalian sera before inoculation into mice

Trichomonad, line 6950♂, was maintained for four days in MDM supplemented with heat-inactivated serum from either a horse, sheep, chicken, guinea pig, rabbit, calf foetus or swine. The cultures were harvested separately and used to infect Balb/c mice, previously

Table 14 The effect of the growth phase of trichomonads *in vitro* upon their infectivity to mice intravaginally.

Number of trichomonads/ ml of culture when harvested[a]	Growth phase	Number of mice infected on day 10/ group size
35×10^4	decline	4/8
82×10^4	late log	8/8
30×10^4	mid log	4/8
100×10^4	early stationary	2/6
40×10^4	mid log	4/6
15×10^4	lag	0/6

a all trichomonad cultures (line 6950) were from a single source and had been cultured for the same time

Table 15 The number of parasites[a] required to produce an intravaginal infection in mice

Number of trichomonads inoculated	Number of mice infected at day 10/ group size
1×10^6	7/8
1×10^5	8/8
5×10^4	5/7
1×10^4	5/8
5×10^3	6/8
1×10^3	6/7

[a] mice were inoculated intravaginally with *T. vaginalis* line 6950 ♂.

injected with estrogen using the standard protocol. The results are detailed in table 16. Trichomonads maintained in all of the sera were capable of producing an infection at 10 days, but trichomonads maintained in foetal calf serum gave a lower infection rate than those maintained in the other sera, although this experiment would need to be repeated to verify this.

3.4. CHARACTERISTICS OF THE VAGINAL ENVIRONMENT THAT AFFECT SUSCEPTIBILITY TO *T. VAGINALIS*

3.4.1. The effect of the use of agar or sterispon in the inoculation procedure

The results described previously suggest that the microenviroment of the mouse vagina is crucial to the susceptibility of the individual to *T. vaginalis*. The parasite inoculum procedure will effect the microenviroment of the vagina, so differences in this could possibly have effects upon the infection rate. The effects using sterispon instead of, or in addition to, agar, and also varying the concentration of agar used in the inoculum medium, upon mouse susceptibility were evaluated. The results (Table 17) suggest that the use of agar as a vaginal plug does not increase the infection rate compared to using no vaginal plug. An experiment comparing the infection rates when using either sterispon or agar showed little difference between the two (Table 17). Agar at 0.5%, however, appeared to be detrimental.

Table 16 The effect of maintenance of *T. vaginalis* in various mammalian sera on the intravaginal infection rate of mice.

Serum[a]	Number of mice infected/group size at 10 days
Horse	6/7
Foetal Calf	1/6
Sheep	4/6
Chicken	4/6
Guinea pig	3/6
Rabbit	6/6
Swine	4/8

[a] Trichomonad, line 6950♂ was maintained in MDM supplement with the various heat-inactivated sera (10% v/v) for four days before being used to inoculate Balb/c mice.

Table 17 The effects of using agar and sterispon upon infectivity of *T. vaginalis* in mice[a]

	Experiment Conditions	Number of mice infected at day 10/group size		
1	No sterispon or agar	3/6		
	0.3% agar	3/6		
2	+ sterispon[b]	6/6	1/6	3/6
	- sterispon[b]	4/6	5/6	5/6
3	+ sterispon [c]	13/18		
	+ 0.3 % w/v agar[a]	16/16		
4	% agar[a]			
	0.2	4/6		
	0.3	5/6		
	0.4	3/6		
	0.5	1/6		

a Sterispon plug was not used in addition to agar at the time of infection.

b trichomonads were suspended in MDM + 0.3% agar for use in infection.

c no agar was present in the infecting inoculum

3.4.2. The effect of frequent sampling of vaginal contents

It was possible that frequent sampling of vaginal secretions would adversely affect infections. This was investigated and the results of the study are shown in Table 18. There was an apparent slight decrease in the infection rates of the groups of mice that had undergone frequent vaginal washouts. Clearly, however, most infections were able to survive interference by the procedure.

3.4.3. Vaginal pH as a determinant of susceptibility to intravaginal infection with T. vaginalis.

Studies were carried out to see if there was a correlation between pH of the vagina at the time of inoculation of parasites and susceptibility of mice to infection. Mice were estradiol-treated, following the standard protocol, or similarly injected with corn oil only. Two days later the vaginal pH was determined. The results (Table 19) indicate that the pH of the murine vagina is normally near neutral and that estrogen does not significantly affect this. Other groups of mice were similarly treated and inoculated intravaginally with trichomonads three hours after the pH of the vagina had been determined. The number of infected mice was determined on day 10 post infection. The results (Table 20) show that pH is apparently not an important determinant in the ability of the trichomonads

Table 18 The effect of multiple vaginal washouts on parasite[a] infections

Conditions	Number of mice infected/group size day post infection		
	day 3	day 7	day 10
CONTROL	-	-	7/20
frequent vaginal sampling	13/20	10/20	4/20
	day 4	day 7	day 10
CONTROL	-	-	1/6
frequent vaginal sampling	3/6	b	1/6
	day 4	day 6	day 10
CONTROL	-	-	8/8
frequent vaginal sampling	8/8	7/8	5/7[a]

- vaginal sampling not performed.

a one infected mouse killed at day 8

b vagina washed out but samples not cultured.

Table 19 Comparison of vaginal pH levels of estradiol-treated[a] and PBS-treated[b] mice

Age of mice (months)	Treatment	Group Size	Number of washouts	pH of vaginal washouts[c] mean	standard deviation
2	Estradiol	6	12	6.8	0.2
3	Estradiol	6	12	6.7	0.3
4	Estradiol	6	14	7.0	0.4
3	PBS	6	15	6.7	0.3

No significant difference ($P > 5\%$) between estradiol and PBS-treated mice.

[a] estradiol treatment carried out following standard procedure.

[b] PBS given subcutaneously, 0.2 ml per mouse.

[c] Vaginal pH was determined two days after estradiol treatment.

Table 20 The effect of vaginal pH on susceptibility of mice[a] to intravaginal infection with *T. vaginalis*

Age of mice (months)	Number of mice infected/group at 10 days	group size	Number of washouts	pH of vaginal washout 2 days after estradiol treatment mean	standard deviation
7	3/6		15	6.66	0.4
5	4/6		12	6.82	0.17
3	2/6		13	6.84	0.44
2	1/6		13	6.93	0.38

Group	Group size	Number of washouts	pH of vaginal washout 2 days after estradiol treatment mean	standard deviation
mice with infection at 10 days	10	23	6.7	0.3
mice with no infection at 10 days	14	32	6.84	0.42

No significant difference ($P > 5\%$) between the vaginal pH levels of mice that were infected at 10 days and those that were not.

[a] Mice were estradiol treated following the standard procedure. The pH of the vaginal secretions were measured 2 days later and 3 hours later mice were inoculated intravaginally with *T. vaginalis*.

to colonise the vagina as there was not a significant difference between the vaginal pH of those mice that became infected and those that did not.

3.4.4. The effect of infection on the vaginal pH

To determine whether infection with T. vaginalis changed the pH of the mouse vagina groups of mice were treated with estradiol and some were intraperitoneally injected one day later with ferric ammonium citrate (100 mg/kg, 0.1 ml), the remainder being given PBS instead. The following day they were all inoculated with T. vaginalis intravaginally. The infection rate at 10 days was determined and 8 days later the vaginal pH values were determined again. The results are shown in Table 21. Statistical analysis of the data revealed the pH of the vaginal washouts of iron-treated mice, was significantly greater than that of PBS-treated mice but that there is no difference between the pH level of vaginal washouts of mice that became infected and those that did not.

A similar experiment was carried out in which the order of manipulations was reversed, that is the vaginal pH was measured at 6 days and infection determined at day 11. Mice had been administered estrogen doses of either 40 mg/kg body weight or 4mg/kg body weight. The results are shown in Table 22. There was no significant difference between the vaginal pH values at 6 days of the groups given different estradiol doses. Statistical

Table 21 Comparison of the vaginal pH of iron-treated and control mice.

Treatment	Number of mice infected/group at 10 days	group size	Number of/group washouts	pH of vaginal washout 2 days after estradiol treatment mean	standard deviation
PBS[b]	5/9		24	7.44	0.59
Ferric ammonium citrate[c] [b]	6/9		22	8.01	0.49

The figures are significantly different at 0.1%

Group	Group size	Number of washouts	pH of vaginal washout at 18 days mean	stadard deviation
Mice with infection at 10 days	11	29	7.8	0.5
Mice with no infection at 10 days	7	17	7.56	0.7

No significant difference ($P > 5\%$) between the vaginal pH of infected and uninfected mice.

[a] Mice were estradiol-treated following the standard procedure.

[b] 0.1 ml of PBS was administered orally one day before inoculation with parasites.

[c] Ferric ammonium citrate was administered as described in the Materials and Methods Section.

Table 22 Comparison of vaginal pH following different estradiol treatments.

Estradiol (mg/kg)	Number of mice infected/group at 11 days	size	Number of washouts/group	pH of vaginal washouts at 6 days mean	standard deviation
40	2/10		24	6.9	0.2
4	5/8		18	7.04	0.27

Vaginal pH of infected and non infected mice at 11 days

Group	Group Size	Number of washouts	pH of vaginal washout at 18 days mean	standard deviation
Mice with infection at 11 days	7	17	6.98	0.26
Mice with no infection at 11 days	11	25	7.03	0.21

No significant difference ($P > 5\%$) between the vaginal pH of infected and non-infected mice.

analysis of the pH values of mice that were shown to be infected on day 11 and those that were not revealed no significant difference, with respect to vaginal pH, between the groups.

3.4.5. Iron levels in the vaginal secretions of mice

It has been reported that iron availability can affect the rate of hydrogenosomal activity of *T. vaginalis* (Gorrell 1985). The effect of various iron sulphate concentrations on the growth of *T. vaginalis* maintained in axenic culture under normal conditions was investigated (Figure 5). Increasing the iron concentrations of the medium up to 100 mg/l, which is the usual concentration in MDM, seemed to accelerate the decline phase of the culture but had little apparent effect on the growth rates or maximum cell number obtained. Surprisingly the parasites grew well in MDM in which all iron sulphate had been omitted. It must be remembered however that the initial inoculum of parasites were suspended in MDM with 100 mg/l iron sulphate which would have resulted in a final concentration of 5 µg/l.

The levels of iron found in the cell free fraction of the vaginal washout (collected as described in the Materials and Methods section) were determined by atomic absorption spectrometry. The results are shown in Table 23. The iron levels of all samples tested were very

FIGURE 5 THE EFFECT OF IRON SULPHATE ON T. VAGINALIS GROWTH IN VITRO.

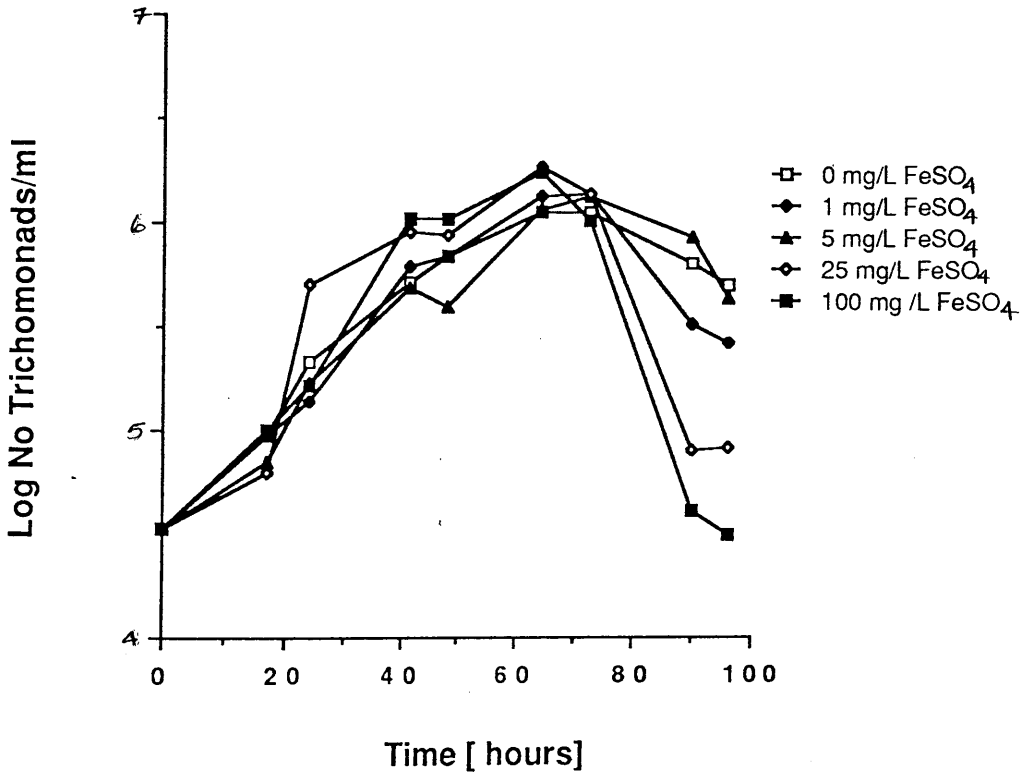


Table 23 Iron content of vaginal secretions[a]

Treatment	Iron concentration detected (mg/l)	
	Experiment 1	Experiment 2
estradiol[bd]	0.08	0.09
ferric ammonium citrate[cd]	0.04	0.15
untreated[d]	-	0.04
untreated[e]	-	-

[a] The vaginae of the mice (20 mice per group) washed out with 0.5 ml PBS or H_2O (as indicated) and all samples in each group were pooled. The pooled samples were centrifuged at 1000 g for 10 minutes and the supernatant removed. The volume of this was 2.1 mls, giving a dilution factor of the vaginal fluid of approximately 2-fold.

[b] estradiol-cypionate administered at 40 mg/kg body weight, subcutaneously, 2 days before vaginal washout.

[c] ferric ammonium citrate administered intrapertioneally at 100 mg/kg body weight (0.1 ml) 1 day before washout.

[d] vaginal washout performed using PBS.

[e] vaginal washout performed using distilled water.

- not done.

low, mostly below the level of accurate detection (0.12 mg/l) of the instrument used. This was the case even when the mouse had been pre-treated with iron. The concentrations detected, even allowing for the approximate 2-fold dilution of vaginal secretions introduced by the washout procedure, are low and this low level of iron available may affect the chances of *T. vaginalis* becoming established.

3.4.6. *Zinc levels in vaginal secretions of mice*

It has been reported that the concentrations of zinc affect the survival of *T. vaginalis*, with high levels being toxic to the parasite. I studied this by determining the effect of zinc on the growth of *T. vaginalis* and by measuring the concentrations of zinc in vaginal secretions. The effect of zinc chloride on the growth of *T. vaginalis in vitro* is shown in Figure 6. Addition of zinc chloride at concentrations of greater than 200 mg/l resulted in cultures growing more slowly and reaching a lower maximum density than control cultures. Cultures with zinc chloride at 500 mg/l grew very slowly, although the parasites were not killed. The zinc concentrations detected in the vaginal washouts are given in Table 24. In all cases the levels were low, some 100-fold lower than the level shown to affect growth of *T. vaginalis in vitro*, even allowing for the two-fold dilution caused by the washout procedure and adjusting the concentrations of zinc chloride to give the levels of the zinc ion.

FIGURE6 THE EFFECT OF ZINC CHLORIDE ON T.VAGINALIS GROWTH IN VITRO.

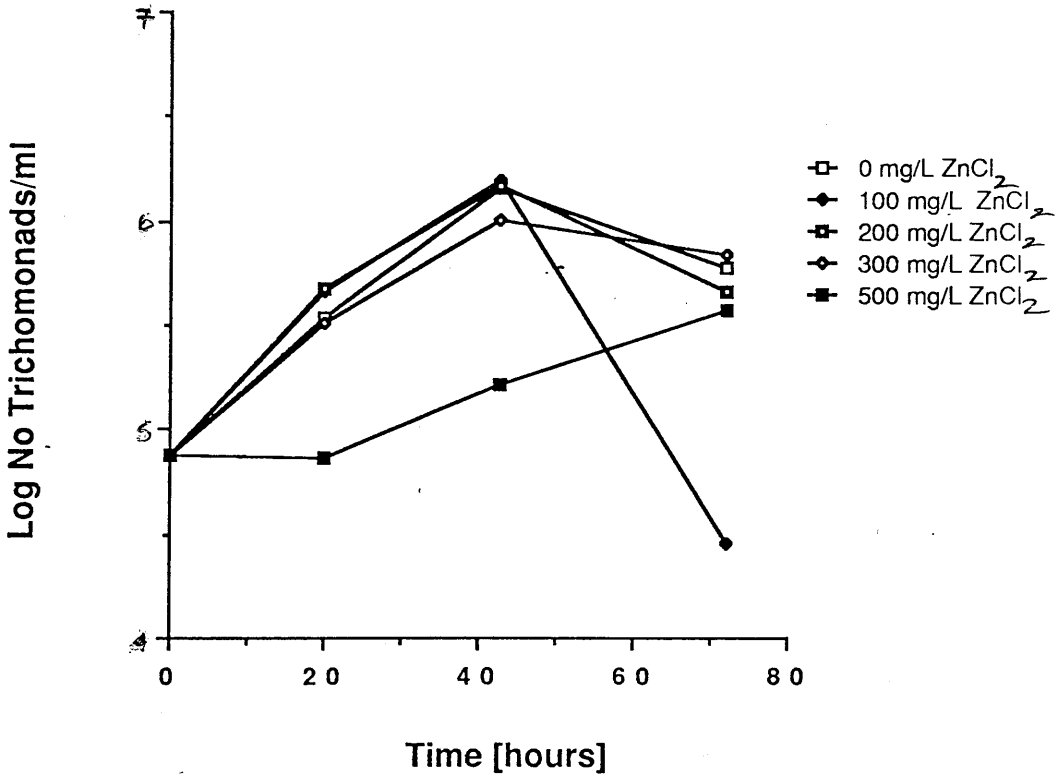


Table 24 Zinc content of vaginal secretions

Treatment	Zinc concentration detected (mg/l)
estradiol[a] [c]	0.4
ferric ammonium citrate [b] [c]	0.2
untreated[c]	0.3

[a] estradiol cypionate administered at 40 mg/kg body weight, subcutaneously, 2 days before vaginal washout.

[b] ferric ammonium citrate administered intraperitoneally at 100 mg/kg body weight (0.1ml) 1 day before vaginal washout.

[c] vaginal washout performed using PBS.

[e] 20 mice in each group.

[f] The vaginae of the mice (20 mice per group) were washed out with 0.05 mls PBS and all samples in each group were pooled. The pooled samples were centrifuged at 100 g 10 minutes and the supernatant removed. The volume of this was 2.4 mls, giving a dilution factor of the vaginal fluid of approximately 2-fold.

3.4.7. The Estrous Cycle

It was implied in the rationale for pre-treating the mice with estradiol that susceptibility of mice to *T. vaginalis* was determined by the stage of the estrous cycle they were at when inoculated with parasites. I investigated whether this was true by determining daily the estrous state of untreated and estradiol-treated Babl/c females. This was carried out by preparation of vaginal smears with subsequent microscopical examination.

Most of those mice that were untreated or given corn oil only, had a fairly regular estrous cycle, with diestrous lasting between one and five days (Tables 25-28). The cycles differed from classical descriptions (e.g. Boot cited in Von Ebberhorst Tergbegon, 1955) in that both proestrous and estrous may last considerably longer than the described 21 hours. However, other authors (Allen, 1922; Parkes, 1928; Snell, 1941) have also observed that the cycle length is highly variable and easily influenced.

Those mice that were given estrogen were generally brought into estrous within two days and the estrous often lasted for a day or two. Subsequent to this though the cycle frequently entered a prolonged diestrous of up to ten days. Inoculation of *T. vaginalis* intravaginally into estrogen-treated females

Table 25 The estrous cycle of normal and estrogen-treated mice

Treatment	Experiment 1 Day 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
estradiol administered on day 3	D	D	P	P	E	E	E	M	D	D	D	E	M	M	M	M	M
	E	D	D	P	E	E	M	D	D	D	D	D/P	D	D	D	D	D
	E	D	D	D	P	E	E	E	M	M	D	M	D	M	D	D	D
	P	P	E	E	E	M	D	D	D	D	D	D	D	D	D/P	P	D
	D	D	D	P	E	E	E	M	D	D	D	D	D	D	D	D	-
	P/M	D	P	P	P	E	E	M	D	D	D	D	D	D	D	D	D
No treatment	M	D	D	P	M	D	D	D	P	E	D	D	D	P	E	D	D
	P	E	D	D	P	E	D	D	P	E	D	D	D	E	M	D	P
	D	D	D	D	P	P	E	D	D	D	P	E	E	D	D	P	P/E
	D	D	D	P	E	E	M	D	D	P/E	E	M	D	D	-	P	P
	P	P	E	D	D	D	P	E	D	D	D	D	D	D/P	D	P	E
	P	E	D	D	P	P	E	D	D	D	P	P	E	D	D	D/P	P

D = diestrous P = proestrous E = estrous M = metaestrous - results unavailable

Table 26 Estrous cycle of corn oil and estrogen-treated mice and mice that had been inoculated intravaginally with *T. vaginalis* 6950 ♂.

	Experiment 2											
	Day											
Treatment	1	2	3	4	5	6	7	8	9	10	11	12
corn oil	D	P	E	M	D	D	-	-	-	-	-	-
administered	N	E	E	M	D	D	D	E	E	M	D	P
day 3	D	E	M	D	D	P	E	M	M	D	D	D
	E	M	D	D	P/E	E	D	D	D	D	D	E
	E	M/D	D	D	P/E	E	D	D	D	P	E	M
	D	E	M	D	-	P/E	E	M	D	D/P	E	M
estrogen	D	D	D	P/E	E	E	E	M	D	D	D	D
administered	D	P/E	E	E	E	E	M	D	D	D	D	D
day 3	D	P	P	E	E	M	D	D	D	D	D	D
	E	D	D	D	P	E	E	E	E	M	M	M
	D	P/E	E	E	E	M	D	D	D	D	D	D
	E	E	E	E	E	E	D	D	D	D	D	D
estrogen	M	M	D	E	E	E	E	M	M	E	E	M
administered	M/D	D	D	P	E	E	E	M	M	M	M	M
day 3 and	P	E	D	D	E	D	D	D	D	D	D	D
T. vaginalis	D	E	E	E	E	M	D	D	D	D	D	D
inoculated	E	M	D	E	E	E	E	E	E	E	E	E
intravaginally	M	D	D	P/E	E	E	E	E	E	E	E	E
on day 5												

D = diestrous

P = proestrous

E = estrous

M = metaestrous

- results unavailable

Table 27 Estrous cycle of estrogen-treated mice and mice that had also been inoculated intravaginally with *T. vaginalis*

	Experiment 3																
	Day																
Treatment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
estradiol administered day 2	-	-	M	-	M	D	-	M	M	D	D	D	D	-	M	D	P
	-	-	D	-	D	D	-	D	D	D	D	D	D	-	D	D	P
	-	-	D	-	D	D	-	D	D	D	D	D	D	-	D	D	D
	-	-	D	-	D	D	-	D	D	D	D	D	D	-	D	D	D
	-	-	D	-	D	D	-	D	D	D	D	D	D	-	D	D	D
estradiol administered day 2, *T. vaginalis* inoculated intravaginally day 5	-	-	E	-	E	E	-	E	M	M	D	D	D	-	D	D	D
	-	-	D	-	P	D	-	D	E	D	D	D	D	-	D	D	D
	-	-	E	-	E	E	-	D	D	D	D	D	D	-	D	D	D
	-	-	D	-	D	E	-	E	M	D	D	D	D	-	D	D	D
	-	-	D	-	P	E	-	E	E	M	D	D	D	-	D	D	E
	-	-	E	-	M	M	-	D	D	D	P	D	D	-	D	D	D

D = diestrous P = proestrous E = estrous M = metaestrous - results unavailable

Table 28 Estrous cycle of corn oil and estrogen-treated mice and mice that had also been inoculated intravaginally with *T. vaginalis* 6950 ♂[a].

	Experiment 4												
	Day												
Treatment	1	2	3	4	5	6	7	8	9	10	11	12	13
corn oil	E	E	M	D	P	E	E	D	D	E	D	P	-
administered	D	D	D	D	P/E	E	E	M	D	D	D	D	D
day 1	D	D	D	D/P	P	P	P	E	D	P	D	D	D
	D	D	D	D	E	E	D	D	D	P	D	P	D
	P	E	D	D	D	M	D	D	D	D	E	D	D
estrogen	D	D	P/E	D	D	D	D	D	D	D	D	D	D
administered	D	D	D	D	D	D	D	D	D	D	D	D	D
day 1	P/E	D	E	E	M	D	D	P/E	P	D	D	D	D

Table 28 (continued)

Treatment	Experiment 4 Day 1	2	3	4	5	6	7	8	9	10	11	12	13
estrogen administered	D	P/E	D	D	P	E	E	E	D	D	D	D	D[a]
day 3 and *I. vaginalis*	D	D	D	D	E	E	M	D	D	D	P	E	E[b]
inoculated intravaginally	E	E	D	D	D	D	P	P	P	D	E	D	E[b]
on day 5	D	D	D	P	P	D	P	E	E	E	D	D	D[b]
	D	D	D	P	E	M	D	D	P	D	E	E	E[b]
	D	P	D	D	P	E	M	D	D	E	M	M	M[b]

D = diestrous P = proestrous E = estrous M = metaestrous

[a] mouse still infected on day 12 post-infection

[b] mouse not infected on day 12 post-infection

- results unavailable.

did not always alter this pattern, with diestrous in some mice lasting up to fifteen days. Other infected animals showed prolonged estrous (up to nine days) or a return to cyclical behaviour. Thus, there appeared to be a variety of patterns of estrous for mice inoculated with parasites and certainly the introduction of parasites in many cases interfered with the 'normal cycle'.

Unfortunately it was not possible to monitor infections at the same time as the estrous cycle and correlations between the establishment of infection and the effect on the estrous cycle could not be made.

3.5. THE SUITABILITY OF THIS MODEL FOR CHEMOTHERAPY, IMMUNOLOGY AND VACCINE EXPERIMENTS

3.5.1. Use of metronidazole

The availability of a positive control would be important if this model was to be successful in assessing the therapeutic value of antitrichomonal drugs. The standard antitrichomonal drug, metronidazole, was found to be extremely effective in this model of vaginal trichomoniasis, when administered orally or subcutaneously. The threshold limit of effectiveness was between 10 and 25 mg of metronidazole/kg body weight, administered once daily over five days (Table 29).

Table 29 The effectiveness of Metronidazole in this mouse model of vaginal trichomoniasis[a]

Conditions of treatment[b]	Metronidazole dose (mg/kg)	route of administration P.O.	C.
Cellacol-treated	-	33/44	6/11
Metronidazole-	10	12/10	2/10
treated	25	0/10	0/10
	50	0/42	0/10

[a] mice were infected with T. vaginalis, line 6950 ♂

[b] Cellacol or Metronidazole were administered daily over 5 days, starting 3 days post-infection.

3.5.2. The efficacy of SolcoTrichovac using this model of vaginal trichomoniasis

The model was used to assess the efficacy of SolcoTrichovac, a newly-marketed vaccine for trichomoniasis (Rippman ET 1982). It was administered to mice either intraperitoneally at the full human dose or intramuscularly giving one-fifth of the human dosage. This corresponds to 7×10^9 lyophilized lactobacilli administered subcutaneously or 1.4×10^9 if administered intramuscularly. These were the doses recommended by the manufacturer. The results are presented in Table 30. They show that there is no difference in the infection rates of the SolcoTrichovac-treated mice and the control groups given only PBS.

3.5.3. Attempts to vaccinate mice with parasite homogenates

To investigate whether parasite homogenates induced any protective immune response, homogenates were prepared by sonication or freeze-thawing T. vaginalis and administered either in Freunds Complete Adjuvant (FCA) or PBS as detailed in the Materials and Methods section. The results from this series of experiments were variable (Table 30). In experiments 1 and 2 there was no increased resistance to infection in vaccinated mice compared to controls while in experiment 3 there was a consistent drop in the infectivity rates of "vaccinated" mice. The mice used in experiment 3 were

Table 30 The efficacy of SolcoTrichovac and parasite homogenate (vaccines) against intravaginal trichomoniasis in Balb/c mice.

Treatment	Route of administration	Number of mice infected/group size on day 10			
		Experiment number			
		1	2	3	4
Solcotrichovac	1P	7/10	5/10		
PBS	1P	9/10	5/10		
Solcotrichovac	1M	8/10	3/10		
PBS	1M	7/10	6/10	2/5	
untreated	-	- (11 day)	8/10	1/5	
		1[c]	2[d]	3[d]	4[ce]
T. vaginalis in FCA	SC	8/10	4/10	5/10	2/5
FCA only	SC	3/10	7/9	9/10	5/5
T. vaginalis in PBS	iP	6/10	8/10	4/10	4/10
PBS only	iP	6/10	7/10	7/10	8/9
T. vaginalis in PBS	SC	5/10	7/10	0/10	4/10
PBS only	SC	5/9	7/10	6/10	7/10

Table 30 (continued)

Treatment		Route of administration	Number of mice infected/group size on day 10			
			Experiment number			
			1	2	3	4
PBS		P-O	7/10			
T. vaginalis	10^7	P-O	6/10			
	10^5	P-O	8/10			
	10^3	P-O	7/10			

[a] all results are for 10 day post infection except where stated otherwise.
[b] vaccination was carried out as detailed in the Materials and Methods section.
[c] trichomonads homogenate were prepared by twice freze thawing until a lysate was obtained.
[e] all mice from 1c were 'cured' using metranidazole and re-infected 18 weeks later following the standard protocol.

IP = intraperitoneally SC = subcutaneously P-O = oral

subsequently treated with metronidazole at a level that would ensure that the infection was cured (as detailed in Materials and Methods) and re-infected a week later using the standard protocol. The results (Table 30, experiment 4) showed that again fewer of the group of mice originally vaccinated with trichomonad homogenates became infected than the control group. This apparent 'long-term' resistance was not demonstrated in all mice that had been resistant to infection in experiment 3.

It is likely that much of the resistance to T. vaginalis infections, as far as it exists which is not confirmed, is mediated by the secretory immune system. In an attempt to establish the importance of this type of response, freeze-thawed trichomonads were administered orally to mice in an attempt to trigger the secretory immune response. A short experiment was designed involving only one oral administration of trichomonads. The results (Table 30) show no difference between the infection rates of mice that were T. vaginalis-treated and mice that were administered PBS.

3.5.4. The effect of re-exposure of mice to T. vaginalis

In order to study further the occurrence of immunity to T. vaginalis inoculated intravaginally mice that had either resolved an earlier infection, or had been inoculated with parasites but had never become infected, were re-exposed to T. vaginalis, using the

standard infection procedure, and examined for infections at ten days. The results (Table 31) show that a failure to infect mice at the first attempt does not prohibit an infection on subsequent attempts. Similarly those mice that resolved the first infection do not necessarily show resistance to further challenges.

It was also shown that subcutaneous infection of mice with trichomonads prior to intravaginal exposure does not increase the resistance of the mouse to an intravaginal infection. Mice were infected subcutaneously in a shaven rump with 1×10^5 trichomonads in 0.16% agar plus MDM plus 10% HIHS and the subcutaneous lesion that was formed was allowed to burst and heal naturally. Two weeks post-infection the mice were exposed to trichomonads by the intravaginal route. It was found (Table 31) that mice that had recovered from subcutaneous infection with *T. vaginalis* demonstrated no acquired resistance to vaginal infections.

Table 3: The effect of re-exposure of mice to *T. vaginalis*

Experiment	Treatment	Number of mice infected/group size at day 10		
		Experiment number		
		1	2	3
1	naive mice[a]	7/10	4/10	8/10
	mice previously inoculated with *T. vaginalis* but never showed infection	8/10	5/10	7/10
	mice previously inoculated with *T. vaginalis*, became infected and resolved infection	8/10	3/6	
2	naive mice[a]	2/4	1/6	
	mice previously infected subcutaneously[b]	6/6	5/9	

[a] naive mice were of approximately the same age as previously-inoculated mice.

[b] mice that had been exposed to *T. vaginalis* approximately four weeks before re-inoculation of the parasite.

DISCUSSION

4.1. SUITABILITY OF THIS MODEL FOR STUDYING TRICHOMONIASIS

The development of a good animal model of human disease relies on fulfilling several criteria. Firstly the aetiologic agent of the animal disease should be the same as that found in human infections. It is important that the infecting organism is capable of producing disease in humans. Secondly the animal disease produced should have characteristics similar to those of the human infection including route of infection, symptoms and duration. Thirdly it must be reproducible.

Perhaps the ideal model would be a naturally occurring infection in a suitable laboratory host that fulfills the above criteria. Unfortunately this is not available for T. vaginalis so these parasites have to be introduced into the animal. The isolates inoculated into the mice were from patients presenting disease at a genitourinary clinic, so they were initially, at least, capable of infecting humans. It is fortunate that T. vaginalis will infect mice as venereal infections tend to be species-specific (Corbeil, 1980), possibly due to the organisms having species-specific receptors for attachment to the vaginal epithelium.

The route of infection of mice is the same as in the human population, i.e. introduction of trichomonads into the vagina, so allowing study of the infection under conditions as similar as possible. The symptoms of the infection in the animal model are negligible,

disease only being detected by observing a vaginal washout, although a heavily infected animal may occasionally produce a white discharge. Although the female disease is described as producing a green frothy discharge, many women are also practically asymptomatic.

The duration of the disease is hard to compare with that found in the human population as the onset of human disease may not be known and the disease is often asymptomatic. However, this experimental model commonly has infections lasting 18 days and infections lasting more than 10 weeks have been recorded by me. An infection of this duration allows time to evaluate the effect of a drug treatment, if used in a chemotherapy study, or to follow the course of disease. Although the infections, unlike those found in human populations, are usually apparently self-resolving it lasts sufficiently long to be of considerable use as a model.

A comparison of this model with other reports of murine intra-vaginal infections shows several advantages of the model I developed. Firstly this model requires much less animal handling than some of those previously reported. Only one estrogen dose is necessary to establish infection compared with the weekly injections advocated by Wildfeuer (1974). My results are therefore in accordance with those of Cappuccinelli et al., (1974) who reported that once the infection is established the parasites survive in the vagina in the absence of subsequent estrogen treatment.

The method of infection involves only a single inoculation of trichomonads which can produce an infection rate of 100%. This compares favourably with that of Cappuccinelli et al. (1974), who found that to obtain this infection rate it was necessary to inoculate the mice on 2 consecutive days. This double inoculation was followed by peritoneal trichomoniasis in one of the six mice, this has not been observed in my work. The single inoculation involves much less time in preparation and animal handling.

Another saving in animal supervision time compared to other reported procedures was that of infecting the animals while conscious. Wildfeuer (1974) inoculated mice while under sodium hexobarbitol narcosis so lengthening the procedure considerably.

Throughout this study the inoculation has been with a pure culture of trichomonads which is an advantage over that chosen by Meingassner (1974). Meingassner inoculated mice with a mixture of Candida albicans and T. vaginalis to study the effect of an anti-trichomonal drug. Chemotherapeutic trials or studies following the cause of infection are much simplified if the infection is of a single organism.

The infection rates obtained during this study compare well with other reports. Neither Landolfo et al. (1981) nor Meingassner (1974) reported on the rates of infection but Cappuccinelli et al. (1974) and

Wildfeuer (1974) obtained results similar to those I observed. The infection rates were variable but as no other worker in this field has published typical infection rates, or the amount of variability that can be expected, then comparison of this model with those of others is impossible. The lack of data from other workers could be due to the small numbers of experiments conducted or the fact that variability of infection rates was not observed by them. It is also possible that any variability that did occur was not reported.

Wildfeuer (1974) reported infections lasting 42 days and the infection rates show a gradual decrease with time. I have observed infections lasting more than 100 days but also find that the number of animals showing an infection decreases over time. There is also agreement on the finding that there is little correlation between the percentage of animals that became infected and the duration of infections that did occur.

Overall, although the model is not ideal, it is fairly suitable for use in chemotherapy studies. The infection is tenacious enough to withstand washout of the vagina as may occur during topical drug application or monitoring of the infection and there is a suitable negative control available. Metronidazole is the drug of choice for most trichomonal infections presented so it is beneficial to have a method of comparing the

efficacy of a proposed drug and metronidazole. Metronidazole cured all infections when given at the appropriate dose level (Table 29). It appeared that the effect was concentration-dependent. It would be of interest to evaluate the threshold at which all infections were resolved. More work needs to be done in this area.

This model has distinct advantages over the equivalent hamster model and rat model. The hamster model has several disadvantages in that they are coprophagic, can cross infect, require more animal handling and, most important of all, harbour a natural trichomonad that can overgrow T. vaginalis. The rat model is also more expensive and requires more animal handling and work done so far (Cavier & Mossion 1956, Meingassner et al., 1975) indicates that rats need to be ovariectomised before use in experiments.

The model is also suitable for studying the host-parasite interaction, for instance the immune mechanism that may operate against the parasite as will be discussed later, and understanding of the pathogenicity of the parasite in vivo.

However, there are problems with this model, mainly its variability and the fact that 100% infection rates were not consistently, or frequently, achieved. These disadvantages mean that it is more expensive to use than would be hoped for, requiring an increased use of

animals and more time to repeat experiments in order to achieve reliable results. Also more work is required to determine why some experiments yield no positive infections at all, as this is clearly a difficulty. The model also does not allow for quantitative studies of trichomonads in the vagina as the method of detection provides data only on presence or absence of parasites rather than degree of infection.

4.2. THE DEVELOPMENT OF THE MODEL: FACTORS IMPORTANT IN ESTABLISHING INFECTIONS.

In my attempt to develop a mouse model of intravaginal trichomoniasis, I tried to identify factors that are crucial to the outcome of the disease. It is clear that the outcome of a parasite challenge is by no means straightforward but depends on the interplay between at least three parameters. One factor is that of the innate variation in the host, such as genetic background, and environmental conditions such as diet and the presence of other infections. A second determinant in the severity of infection is the extent of the challenge to which the animal is exposed. A third parameter is that of variation in pathogenicity of the infecting organism. The results section was, for ease of presentation, divided into sections on host characteristics, parasite characteristics and the vaginal environment. The discussion will also follow

this format, but it must be remembered that they are not exclusive but together influence the course of disease.

It is clear from the results presented and published work that the physiology of the host is crucial in determining susceptibility to Trichomonas vaginalis. One way in which susceptibility was modified was by estrogen treatment.

The need for estrogen treatment of mice prior to infection with trichomonads has already been reported by many authors (Landolfo et al., 1981; Cappuccinelli et al., 1974, Wildfeuer, 1974) and our results have confirmed this finding. The reason for this prerequirement of estradiol is unclear, although many explanations have been proposed. Kupfenberg et al. (1948) suggested that estrogen potentiated infection by enhancing vaginal cell glycogen levels and so increasing food sources available to the trichomonads. They also found that estrogen did not have a direct effect on trichomonads. This was confirmed by the finding of Cappuccinelli et al. (1974) that estrogen is important only in the initial stages of the infection. If the effect was direct rather than mediated then it would be expected that administration would need to continue during the entire course of infection. Another proposal for the effect of estrogen treatment was that it prolonged estrous and so prevented the appearance of the polymorphonuclear leucocytes into the vaginal lumen.

This is the proposal by Wildfeuer (1974) who recommended that mice should be given estrogen at weekly intervals so that they were maintained in estrous. This is clearly not necessary however, as Cappuccinelli *et al.* (1974) and my results show; the parasites can survive for long periods in the vagina without the presence of exogenous estrogen subsequent to the initial dose. Indeed, although polymorphonuclear leucocytes have been reported to demonstrate trichomonicidal activity *in vitro* (Rein *et al.*, 1980) it does appear that their presence *in vivo* is not by itself sufficient to lead to the eradication of parasites. Trichomonads and polymorphonuclear leucocytes were often present in washouts from an infected mouse on successive occasions. If trichomonads could not survive *in vivo* with the leucocytes then they would have disappeared by the next washout. However, it is probable that it is beneficial if polymorphonuclear leucocytes are absent initially in order to establish infection. Certainly it was found that the washouts in which trichomonads appeared first generally were those containing few leucocytes but many epithelial cells.

It is clear that estrogen treatment of mice did bring them into apparent estrous. Changes in bacterial flora during the estrous cycle and associations between *T. vaginalis* and bacteria have already been mentioned as important factors and it is possible that trichomonads will only become established in the vagina in the

presence or absence of certain bacteria. It may be postulated that if these bacteria were only present or absent during estrous then the need for estrogen treatment, prior to infection, could be explained. Larsen et al. (1977) suggested that the cyclical changes in bacterial flora are caused by the presence or absence of a growth factor secreted/excreted by effete epithelial cells. It is conceivable that trichomonads have a requirement for the presence of this growth factor before they can become established in the vagina.

It is possible that estrogen treatment has an effect unrelated to that on the estrous cycle but is crucial in enabling T. vaginalis to establish; an effect on the immune status of the host could fall into this category. It has been observed that estradiol treatment (1μg/day, route not stated), prior to immunisation of ovariectomised rats with sheep red blood cells, via the Peyers patch (part of the secretory immune system), results in greatly decreased IgA and IgG antibody levels in the vaginal secretions (Wira and Sandoe, 1987). The therapeutic effect of estrogen could thus be to inhibit the immune response versus T. vaginalis. It is likely that there is more than one mechanism of action of estrogen and that the effect observed is probably a combination of those mechanisms mentioned above.

The administration of estrogen seems to be relatively unimportant with regards to route, timing and

amount. There was little difference in the effects of subcutaneous, combined subcutaneous and intraperitoneal, and intraperitoneal administration. The subcutaneous administration of estrogen became the chosen method because it involved only one injection and was found to be easier and safer than intraperitoneal inoculation, as a wide bore needle was necessary for use with the oil suspension. The timing of estrogen administration before inoculation of parasites was also found to be relatively unimportant in the range of one to three days. It was considered that due to the nature of the light/dark cycles and their critical influence on the timing of ovulation (Braden, 1957) that there might be a difference in susceptibility to infection between groups that had estrogen administered during the morning or the afternoon. However, this hypothesis proved to be incorrect (see Tables 3 c, d).

The amount of estrogen usually administered to the mice (40 mg/kg body weight) is considerably more than the physiological level needed to initiate estrous. Indeed it is probable that even the lowest dose used (0.05 mg/kg body weight) is well above the threshold level, which may explain why reducing the level to this amount had little effect on the results. It was thought though that these low levels may have a different effect to the high levels on the general physiology of the mice, and in so doing increase their susceptibility and so infection rates. In particular Cappuccinelli et al.

(1974) had reported that cervical secretions rich in estrogen may be capable of preventing parasite adhesion to the epithelium.

As already mentioned the establishment and course of an infection is probably dependant on the interplay between two, or more, parameters. For this reason experiments that assessed the effect of two variables together (route and timing of estrogen treatment) were performed. In this experiment (Table 3e) no relationship between the parameters was indicated.

One period of unusually low susceptibility of mice to infection followed on from when there had been a change of diet given to the mice. To determine whether the diet change was the cause of a change in the mouse physiology, having a subsequent effect on infection rates, mice were weaned onto either the original diet (41B) or the new diet (CRM). It appears from the results (Table 6), however, that neither of the diets significantly impaired the susceptibility of the mice.

Determination of the effects of genetically-induced variation in physiology on the susceptibility of the mice to T. vaginalis is of considerable interest. Of especial interest are those changes that accompany the aging process, as these have been shown to be critical in determining susceptibility to other diseases. The situation with respect to a parasite of the vaginal tract is a special case, as the environment changes

appreciably at certain times of life. Studies on the prevalence of human trichomoniasis indicate that age is of great relevance as very few prepubertal girls show infection. This may partially be explained by epidemiology and partially by the fact that T. vaginalis cannot settle in prepubescent girls due to a lack of estrogen, causing an absence of glycogen in the vaginal mucous membrane (Peter, 1945). The age of the murine host, however, appears to be of much less relevance. Our results indicate no difference in susceptibility to infection of mice between eight weeks (mice become sexually mature at 4 weeks of age (Bronson, Dagg & Snell, 1966)) and one year. This may indicate that there is truly no difference or that the effect ageing has on the hormonal support of the vagina was abrogated by the use of exogenous estrogen. However, the viability of trichomonads to infect non-estrogen treated mice could relate to Peters finding (1945) that trichomonads need estrogen to provide glycogen in the vagina.

These results are in contrast to those of Landolfo et al. (1981) who reported that susceptibility to T. vaginalis varied with age, even in estrogen-treated mice, being maximal at three to four weeks and minimal at forty to forty two weeks. It was not possible to determine the susceptibility of sexually immature mice as the vagina only opens at the onset of maturity.

The importance of genetic background in determining susceptibility to T. vaginalis was studied by using

several breeds of mice. All strains I used were found to be susceptible to infection, but in varying degrees. Landolfo et al. (1981) similarly observed that C3H mice were less susceptible than Balb/c and DBA but they also reported that C3H and C57Bl mice were completely resistant to intravaginal infection. My results show that C57Bl can be infected. Trichomonads are also capable of infecting Swiss/T mice (Cappuccinelli et al., 1974) and NMRI mice (Meingassner, 1977). The high rate of infections obtained with CFLP is interesting because this is an outbred mouse which therefore has a more variable genetic background. An outbred mouse could be useful in chemotherapy studies where the variable genetic background of the experimental animal group would more accurately mirror the genetic spectrum found in a normal, random human population.

A general finding that not all mice in a group became infected, and that the percentage infection rate varied between groups even of the same inbred strain of mice, prompted me to see if susceptibility was a long-lasting or permanent characteristic or a relatively ephemeral one.

An experiment was designed to evaluate differences in susceptibility between groups of mice that arrived at different times and to see if these differences were maintained (Table 11). Four of the six groups gave no infections while one group yielded a moderate infection

rate and one group showed a high infection rate. These differences cannot be attributed to differences in source of the mice as most were from a single source, including the group with a high infection rate and the groups with no infections. Differences in age are unlikely to be the cause as previous experiments showed age to be unimportant and the two oldest groups in this experiment, although near each other in age, gave infection rates of five out of six and none out of six.

Mice from two of the original groups, from which mice used in experiment one were taken, were infected approximately a month later, again using the standard procedure. The infection rates from this experiment were much higher, with the groups that had no infections in experiment one, having high infection rates in the subsequent experiment. The resistance to infection that was observed the first time was therefore temporary or, less likely, specific to the first group of mice. A change in the infecting trichomonad culture is a possible reason for this increase in infection rates, but is an unlikely explanation because subsequent experiments show little difference between cultures that had been passaged from mice to culture over a year and cultures that were fresh from stabilate. Also the first culture, although not producing a high infection rate in most groups of mice, did give a result of five out of six infected for one group. The culture cannot

therefore be described as lacking virulence. The differences in infectivity of parasite populations is discussed below.

It is difficult to understand why these experiments should yield such different results when the experimental procedure was controlled as far as possible. One suggestion is that of differences in vaginal microenvironment, possibly in relation to changes in the flora. The possible role of bacterial colonisation of the vagina and its relationships with T. vaginalis will be discussed in more detail later.

The characteristics of the parasite isolate play an important part in influencing the outcome of the infection. Differences in virulence of parasite lines have been reported for many other diseases including Leishmania (Scott, et al., 1983), malaria (Walliker et al., 1976) and trypanosomes (Clayton, 1978). Isolates of T. vaginalis have been shown to differ in pathogenicity when investigated using cell culture (Farris and Honigberg, 1970), intraperitoneal infections (Teras and Roigas, 1966) and subcutaneous infections (Honigberg et al., 1966). Winston (1974) reported a relationship between parasite size and pathogenicity for T. vaginalis. However, the pathogenicity index in this instance was determined from patients' records, which takes no account of differing susceptibilities between the women. It is also possible that size of the

parasite depends on the conditions in which it resides and has not a direct relationship to the inherent pathogenicity of the parasite.

The work presented here shows there is no apparent difference in pathogenicity between lines 6950♂ and 39. The results detailed in Table 12 show that lines 6950♂ and 39 caused similar infection rates, although those with line 39 varied more between passages. It is not possible from this experiment, however, to decide whether there is a spectrum of pathogenicity, or if most T. vaginalis are of relatively uniform virulence. More studies, with a range of isolates, are needed. Comparison of several isolates, however suggested that virulence does vary (Coombs et al., 1986).

It is interesting to note that line 6950♂ was originally isolated from a symptomatic male. As has already been mentioned, males that are infected with T. vaginalis are usually symptomless, so it is possible that either line 6950♂, and by inference also line 39, is unusually pathogenic or that the male was very sensitive to trichomoniasis.

The stability of pathogenicity has already been questioned. Laan (1966) observed that the pathogenicity of T. vaginalis, assessed using pathological changes induced in white mice by intraperitoneal infection, remained essentially unchanged on short-term passage in vitro for up to four months, but that it decreased

thereafter. He also reported that the virulence increased with animal passage. Ivey (1975) reported a drop in the virulence of *T. vaginalis* that had been maintained *in vitro* for three months, the method of assessment of virulence again being intraperitoneal injection of mice with parasites.

To provide further information on this aspect, I compared the infectivity of a line of *T. vaginalis* maintained by serial passage intravaginally with the same original line maintained axenically *in vitro*. My results differed from those of Laan (1966) for I found very little difference, or perhaps a slight drop in virulence, of those trichomonads that had been passaged through animals for one year compared to the original culture. There was only a slight drop in infectivity when trichomonads were maintained axenically *in vitro* for 3 months; the drop in infectivity is probably not significant. Thus my results indicate that infectivity was relatively stable with this line. It would be interesting to evaluate the effect of a longer period of maintenance *in vitro*, especially as other work in this laboratory suggested that maintenance for as long as seven or ten months resulted in an almost total loss of infectivity (Coombs *et al.*, 1986).

The reasons for the reported loss of virulence with time in culture are not understood, but it has been suggested that the presence of penicillin and

streptomycin may be factors (Stabler et al., 1967; Honigberg et al., 1970). Such changes, however, have also been reported for other parasites and probably are simply a result of the selection of lines well adapted to the non-physiological conditions in vitro.

The failure of line G3 to infect mice also suggests that long-term culture in vitro can lead to loss of infectivity. Nevertheless it has been shown that line G3 has a 'marked, rapid effect' on mammalian cell lines (Bremner et al., 1986). This suggests that pathogenicity in mice is determined by more than simple pathogenicity towards mammalian cells and that the latter is not a good indication of virulence. Line G3 is a clone from a line that has been maintained in vitro since the 1950's. It would appear that it has either lost its infectivity towards mice during this time, or less likely, was never infective for mice. It was not possible to obtain an original stabilate to compare its pathogenicity.

I was able to confirm, however, that trichomonads preserved in liquid nitrogen retain most of their original virulence, as was previously reported by Diamond (1965). The results also agree with those of Ivey (1975) that trichomonads that have not undergone a long period of in vitro cultivation, and therefore possible selection or stabilisation of virulence, before cryopreservation still retain their virulence.

Conditions under which T. vaginalis were maintained could have profound effects on its pathogenicity. One of the factors considered was the growth phase of the parasites at the time of infection. The preliminary results obtained (Table 4) show that even an overgrown culture in the decline phase can infect mice. However, no infections were produced with parasites from the lag phase of growth.

Trichomonads were also grown in various sera before inoculation into the mice (Table 16). Trichomonads grown in the various heat-inactivated sera (horse, calf, sheep, chicken, guinea pig, rabbit and swine) were all capable of establishing an infection although trichomonads grown in foetal calf serum gave noticably lower infections rates. This experiment needs to be repeated but is potentially of interest. Peterson and Alderete (1984) have already demonstrated that T. vaginalis undergoes absorption of plasma proteins, and they speculated that this is to evade the host immune response. It is possible that these key proteins are present in the sera of older animals but not in foetal calf serum.

The importance of the number of trichomonads in the inoculum was also investigated and the results show good infection rates can be achieved even with as few as 10^3 parasites - 86% of mice inoculated with 10^3 parasites were still infected on day 10. My results contrast with

those reported by Wildfeuer (1974) and Capuccinelli *et al.* (1974). Wildfeuer reported that of the mice inoculated with 1.25×10^6 trichomonads, 67% were infected at 7 days. At the same time only 15% of the mice inoculated with 1.5×10^5 parasites became infected. Cappuccinelli *et al.* found no relationship between parasite inoculum and number of animals infected, although they reported that the minimum infecting dose was 5×10^3 trichomonads.

It is difficult to explain the differences between results as the methods used were not the same, nevertheless it does suggest that the experimental model I developed was more sensitive than the others. It is not known whether there is a minimum infective dose of *T. vaginalis* in humans.

There are many other variables that could be evaluated in order to understand fully the parasite characteristics that play crucial parts in determining the outcome of the infection. Such experiments might include use of more strains, which would be particularly interesting if the original clinical pathology was known, the use of different culture media and the effect of maintaining the trichomonads at various pH values before inoculation. It might also be of interest, given that a relationship between various bacteria and trichomonads has already been established, to inoculate the vagina with a mixed culture of various bacteria and *T. vaginalis*.

The microenvironment of the vagina is also an important consideration in establishing a model of trichomoniasis. It must be acceptable to the trichomonads in order for them to establish an infection and, just as importantly, it must maintain those conditions so that the infection continues over time. It was initially thought that a 'plug' would be helpful in containing the trichomonads in the vagina and to test this agar and sterispon were both investigated. The results in Table 17 suggest that neither resulted in consistently higher infections. I did not, however, carry out many experiments investigating the advantages of plugging the vagina and so these results need to be interpreted with caution. Other authors have used Spongostan (Meingassner, 1977) to avoid loss of inoculum, and Wildfeuer (1974), as already stated, administered the inoculum to mice under sodium hexobarbitol narcosis, presumably to stop the animals moving and the inoculum being forced out of the vagina.

The procedure of washing out the vagina to assess the presence of trichomonads may have had an effect on the longevity of infection, decreasing the infection rate. Adverse effects could have been due to the disruption of the vaginal microenvironment, either by pieces of the epithelium becoming detached or other changes caused by the introduction of the new medium into the vagina; this could affect the colonising trichomonads. In an attempt to determine if a decrease in infection

rate did indeed occur under these conditions it was found that in 2 of the 3 experiments carried out there were higher infection rates in the control group receiving less washouts. The difference is not statistically significant, however, and these experiments will have to be repeated before a firm conclusion can be reached. Nevertheless, the experiments show that frequent washing out does not result in loss of infection in all cases.

Although this method of detecting the infection may cause the loss of some infections after the first washout, it does allow most infections to continue and, given the lack of other suitable detection methods, it is the most reliable method currently available. If the washout is performed identically in studies comparing infection rates, then the possibility of a drop of rates is rendered unimportant. Overall it is a quick, easy method of detecting infections that does not seem to greatly impair the vaginal microenvironment with respect to the trichomonads.

There are many other factors of the vaginal environment that could affect the ability of the trichomonad to colonise. Glycogen levels in the vaginal epithelial cells have already been mentioned as being important in the establishment and maintenance of infections in humans and it would be interesting to monitor the importance of this on a mouse model of

infection. Another area which is probably crucial is the presence or absence of bacteria and other microorganisms in the vagina. Bacteria have been observed to influence the growth of T. vaginalis in culture, even so far as the presence of Staphylococcus aureus prolonging the life of the culture beyond that of bacteria free controls, whereas others greatly decreased multiplication (e.g. Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis) (Pray, 1952). Bacteria can have a considerable effect on the vagina in terms of waste products, causing changes in pH, and there may be competition in the vagina for metabolites, and/or niches. Information on the flora of the murine vagina is not available, so it is not possible to find if the bacterial species known to affect the growth of T. vaginalis are likely to be present during infection. However, P. mirabilis is common in the rat vagina and is therefore possibly common in mice and Staphylococcus is isolated infrequently from rats (Yamada, et al., 1983).

A short study involving local application of antibacterials penicillin, streptomycin (at two or three times the levels normally used in cultured media) or systemic administration of antibacterials penicillin, streptomycin, erythromycin, neomycin, gentamycin (at three times the levels, pro rata, normally used in human bacterial infections) at time of estrogen administration and inoculation of parasites yielded no positive relationship between use of antibacterials and the

susceptibility of the mouse to infection (results not shown). As the resources were not available to determine if the chosen antibotics were removing certain bacteria from the vaginal flora it cannot be decided, at present, whether the antibiotics were having no measurable effect on the bacteria present or if the presence of bacteria does not influence the murine trichomonad infection. Nevertheless this is an area of study of great potential interest.

4.3. THE VAGINAL MICROENVIRONMENT

Comparisons were made of the normal murine vagina and vaginae under various physiological stresses, such as estrogen and iron treatment, in an attempt to gain more information on both the effect of the preinfection procedures and the conditions in the vagina conducive, or refractory, to infection with T. vaginalis. The parameters that were studied were pH, iron and zinc content of vaginal fluids, and the estrous cycle. The effects of infection upon these parameters were also studied to gain further insight into both the conditions in the vagina favourable to trichomonad proliferation and how the infection changes the vaginal environment.

4.3.1. Vaginal pH

It was thought, that the pH of the vagina may be an important factor as to whether a favourable

microenvironment is provided for colonisation by the trichomonads. As stated earlier, it has been reported that the pH of the rat vagina is near neutral and does not fluctuate with changes in the estrous cycle (Blandau et al., 1958; Larsen et al., 1976) whereas the female vagina is more acidic, with cyclical changes in pH according to hormonal support (Wagner and Otteson, 1982). It was of interest to discover which of these models the vaginal pH of the mouse was most like with the idea that, if necessary, I may attempt to change the pH to offer a more favourable environment to the trichomonads.

Results detailed in Table 19 show that the vaginal fluid was near neutral pH whether from estrogen-treated mice, at either 4 or 40 mg/kg body weight, (i.e. in apparent estrous) or untreated mice. The importance of estrogen is therefore probably not due to an effect on the vaginal pH. The pH values of the vaginal fluids of different groups of mice (arriving in the department at different times) were also compared. Four groups of mice with an age spectrum of 13 weeks to 31 weeks were estrodiol-treated, the vaginal pH taken 9 days later, and they were then intravaginally infected with T. vaginalis. The pH values for all the groups of mice were very similar at around pH 6.8, regardless of how many mice in any group became infected. When the mean of the vaginal pH values were compared for mice that became infected and those that did not show an

infection, there was no significant difference between the two groups. It appears from these results then that the pH of the vagina at the time of parasite inoculation does not affect the susceptibility of mice to infection.

The vaginal pH was also measured during infection to assess if there was a difference between the values for mice with an infection and non-infected mice. In addition, the mice had been either previously treated with ferric ammonium citrate or PBS, because, as detailed already (Table 7), iron seems to have a beneficial effect on the infection rates. It was decided to determine if this was due to change in the pH of the vagina. Although the results show a significant decrease in the vaginal pH of iron-treated mice, it can be seen that there is no difference in pH of infected and uninfected mice. The increase in susceptibility to T. vaginalis that iron treatment confers on mice cannot therefore be due to pH alone.

Both experiments involving monitoring the vaginal pH of mice and the presence of T. vaginalis are open to criticism. The mice were either examined for infection at 10 days and the pH monitored 8 days later, or the pH measured at 6 days and the number of infected animals evaluated 5 days later. Both procedures severely disrupt conditions in the vagina during the first examination, which is why it was decided to allow the vagina several days to re-establish the normal

environment. However, it is possible that conditions could deteriorate during this time, possibly with the loss of the infection or a change in the pH. However, other experiments indicate that the infection is unlikely to be lost (see Table 19). An alternative design of the experiment to the ones carried out would be to execute the second examination immediately after the first. It was decided that this would be less favourable as the second washout would be abnormal giving unreliable readings. It is not clear how, with the currently available techniques, experiments such as these can be carried out without these associated problems.

Perhaps what would be the most appropriate method was not available to me, given the equipment available. The ideal experiment would involve determining the pH value and detecting T. vaginalis in the same washout or evaluating the pH level in situ by inserting an electrode into the vagina (as performed by Wagner and Otteson, 1982 and Parsons et al., 1977) immediately prior to the washout procedure.

Overall the results show that the murine vaginal pH, as has been demonstrated with the rat, does not fluctuate with hormonal support. It appears that pH is not important in determining the probability of an infection establishing itself and the presence of an infection does not seem to affect the vaginal pH.

4.3.2. Zinc levels in murine vaginal secretions

As stated earlier, males seem to be more resistant to trichomoniasis than females and this could possibly be attributed to the presence of zinc in the males lower urinary tract. It has been identified as the prostatic antibacterial factor (Fair et al., 1976) and has a broad spectrum, its range including potential genitourinary pathogens such as Candida albicans, Chlamydia trachomatis and gram-positive and gram-negative bacteria. It has also been reported that canine prostatic secretions kill Trichomonas vaginalis possibly due to a high level of zinc (Krieger et al., 1982). The blood zinc level may also be of importance as Willmott et al. (1983) reported the cure of a patient with recalcitrant trichomoniasis by administration of zinc.

It is clear therefore that a study of the zinc levels in the murine vagina would be of interest especially if this was compared to the level of zinc that is trichomonocidal. Krieger and Rein (1982) had found most trichomonad isolates tested were killed at a concentration of more than 1.6 mM zinc chloride (= 0.46 g/l). My results show this level (0.5 g/l) to be inhibitory but not lethal. However, a comparison of the zinc concentration that is inhibitory to T. vaginalis and that found in the mouse vagina using atomic absorption spectrometry (0-0.38 mg/l), even allowing for dilution factor of 2, reveals that zinc is probably not

at a sufficiently high concentration to affect the infection rates.

4.3.3. Iron levels in murine vaginal secretions

My results (Table 7) indicate that the administration of iron to mice, before intravaginal inoculation with trichomonads, does increase the infection rate. It is also responsible for a change in pH of the vagina, but this physiological modification alone does not seem to influence the probability of establishing an intravaginal infection. From these results, it is difficult to determine the mechanism of increasing the infection rates or whether iron has a direct or mediated affect on parasite survival in the vagina.

The effect of increasing the iron content in the media was evaluated and the results agree with those of Gorrell (1985) who found that a 10-fold increase in the iron concentration does not affect the growth rate *in vitro* in axenic culture, or maximum cell density obtained. My results show even a 100-fold difference had little effect. Iron does, however, have an effect on the hydrogenosomal activities, particularly pyruvateferredoxin oxidoreductase (Gorrell 1985, Peterson and Alderete 1985).

The amount of iron in the vagina assessed by atomic absorption spectrometry was found to be very low,

regardless of whether the mice had been ferric ammonium citrate-treated or not. The trichomonads are therefore going from a medium of high iron concentration (100 mg/l) to an environment of low iron concentration (less than 3 mg/l) when they are inoculated into the mouse vagina.

There are at least three possible explanations for the observation that ferric ammonium citrate treatment increases infection rates without increasing the presence of iron in the vagina. Firstly, iron does not have a direct effect on parasite pathogenicity but instead alters the susceptibility of the mice to infection. Iron has already been shown to affect the vaginal pH and it may influence other physiological mechanisms making the vagina a more favourable environment for T. vaginalis.

Secondly, the iron in the vagina is not being recovered by a simple washout procedure. If the iron was present in deeper layers of the epithelium that are not yet ready to be sloughed off then it is unlikely it would appear in the vaginal washout. The iron may be available to trichomonads at this time because of possible attachment of the parasites to the vaginal mucosa.

Thirdly, and perhaps most likely, is that the iron has not yet moved from the peritoneum and entered the vagina. The iron content of vaginal washouts was assessed only 24 hours after administration of iron to

the mouse; it would be interesting to measure the iron content a few days after administration. It is possible that trichomonads survive in the vagina for a few days regardless of iron content, but after this time the survival decreases unless iron then becomes available.

Intraperitoneal administration of iron has a significant effect on the course of an intraperitoneal infection of mice with Tritrichomonas foetus. Budilova & Kulda (1977) reported increased virulence of T. foetus when ferric ammonium citrate was administered at 25-200 mg/kg body weight, for 6 days after intraperitoneal infection. However, their experiment did not distinguish between increasing susceptibility of mice and increased virulence of trichomonads.

Binding of iron to T. vaginalis has been reported (Peterson and Alderete, 1985). The authors found that trichomonads have low affinity receptor sites for a host molecule lactoferrin. Lactoferrin is a protein produced by the mucosal surface of the vagina that has a high affinity binding site for ferric irons. The binding of lactoferrin to T. vaginalis results in intracellular iron accumulation. The authors speculated that the binding of host macromolecules such as lactoferrin, lipoproteins (Peterson & Alderete 1982, 1983, 1984) and others contribute to the parasitic capabilities of the protozoan. It seems likely therefore that the effect of iron treatment on survival rate in the vagina is direct

rather than mediated. More work is needed in this area, particularly a study on the concentration of iron over time in the vagina of iron-treated mice. Information of this sort may lead to more effective changes in the inoculation procedure such as adding iron to the inoculation medium or giving mice smaller but more regular iron treatments.

4.3.4. The Estrous Cycle

The estrous cycle is generally described as being of 4-5 days length composed of a short proestrous and estrous (12 hours each) and a longer metestrous (21 hours) and diestrous (57 hours) (Bronson et al., 1966). However, many observations since then have cast doubt on the validity of characterising a normal estrous cycle.

The results presented here concerning the cycle of untreated mice show a prolonged diestrous period in many cases and this is in agreement with findings of Whitten (1959) and Marsden & Bronson (1965). These workers found that estrous is suppressed when mice are crowded into all female groups and this has been related to olfactory-mediated stimuli.

I found that treatment of mice with estrogen largely disrupts the cycle, mice usually entering estrous approximately 2 days later. The cycle usually then entered diestrous for up to 10 days. If the mice were then infected there seemed to be 3 major patterns

that the estrous cycle followed. The mice either entered a prolonged diestrous of up to 15 days or entered a prolonged estrous or returned to normal cycling. None of the mice that returned to normal cyclical behaviour were infected at day 10 but one mouse that had entered the prolonged diestrous still was infected at this time. The presence of large numbers of neutrophils in the vagina does not therefore seem to prevent trichomonad survival.

The fact that there is great variability in response to estrogen treatment or infection implies that this may be a factor in the variability of infection rates from week to week. It would be of value to repeat these experiments to gain more data on the effect of the estrous cycle on infection duration. Some other work in this laboratory indicates that there is a correlation between stage of estrous and susceptibility of mice to intravaginal infection, mice being most readily infected during early proestrous (Coombs et al., 1986) which is a time of elevated hormone levels and therefore decreased levels of antibodies in the vaginal secretions (Wira and Sandoe, 1987).

Further work in this area should include assessment of the estrous cycle by the appearance of the vagina (Champlin et al., 1983), as frequent vaginal sampling has been reported to result in an abnormally high incidence of cornified smears (Emery and Schwabe, 1956;

Wade and Daisy, 1935) which possibly confuses the results a little.

I used the model I had developed in an attempt to determine location of the trichomonads in the vagina. One aim was to see if they inhabit particular niches with special microenvironments. Mice were given a gentle vaginal scrape with a round-ended implement and the vaginal material obtained immediately examined for the presence of T. vaginalis. If the trichomonads were present then the mouse was sacrificed and the vagina dissected out. This was frozen and sectioned on a cryostat and sections so obtained were stained using acridine orange (Fripp, Mason and Super, 1975) or Papanicalaou (1954) or May-Grunwald-Giemsa (Cook, 1974). Alternatively the vagina was prepared for scanning electron microscopy by either critical point drying or freeze-drying. These procedures were performed on vagina that had either been gently washed with PBS (to remove mucus) or unwashed vaginae.

None of these procedures, however, revealed the presence of trichomonads (results not shown). The staining of sections, using May-Grunwald-Giemsa or Papanicalaou, for use in light microscopy did not distinguish between T. vaginalis and mammalian cells, while those stained with acridine orange did not show stained trichomonads in sections. Scanning electron microscopy did not reveal any trichomonads, possibly

because of the washing procedure. However, this procedure was found to be necessary as specimens left unwashed were covered in layers of mucus, obscuring detail underneath. While it is possible that the random selection of sections examined did not accurately reflect the true presence of trichomonads in the vagina, it is more likely that this finding indicates that T. vaginalis is not in high numbers in the murine infection and any attachment it does make to epithelial cells is weak or that the cells are easily sloughed off with the trichomonads from the vagina.

4.4. THE IMPORTANCE OF THE MURINE IMMUNE RESPONSE TO INTRAVAGINAL TRICHOMONIASIS.

SolcoTrichovac (STV) is currently marketed as a vaccine against urinogenital trichomoniasis. It is prepared from inactivated, aberant strains of Lactobacillus acidophilus which the makers of STV propose share antigens with T. vaginalis. They suggest that the vaccine induces cross-reacting antibodies versus the abnormal lactobacilli and T. vaginalis. They also suggest that this does not adversely affect the normal lactobacilli (Stoikovic, 1982).

When this vaccine was tested in the mouse model, following the protocol suggested for human trichomoniasis (which involved therefore an approximately 3000 times greater dose per body weight

than used in human treatment), or reducing the dose by one-fifth, there was no difference in the infection rates between the vaccinated and unvaccinated animals. It is possible that the lactobacilli antigens in the vaccine preparation do not cross react with T. vaginalis antigens so that an immune response with an activity towards trichomonads is not triggered. This explanation is favoured by Gombosova et al (1986) who reported that there is no evidence of antigenic similarity between the two organisms using immunofluorescence, agglutination or haemagglutination assays. These results are not surprising given that the organisms are so unrelated. The authors speculate that the immuno-therapeutic effect of the vaccine (Goisis et al., 1982; Ripman 1982) is due to stimulation of the non-specific response. Alternatively it is possible that the vaccine did not work in this model because the antigens were not presented in such a way as to trigger an immune response.

I also attempted vaccination of mice with sonicated or freeze-thawed trichomonads. The 'vaccine' dose was derived from between 1-5 x 10^6 trichomonads. This would have contained 0.1 - 0.5 mg protein (Gorrell, 1985). The amount of immunogen necessary to initiate a protective immune response in the host is not known, but these amounts ensured that the vaccine would contain more than the low-tolerance limit (that is the specific non-

reactivity of the lymphoid tissue induced by administration of very low levels of antigen).

Nevertheless, vaccination using this procedure had no apparent affect on the infection rate. Clearly there was no protective immune response stimulated. The development of vaccines is a complex process and the effect produced depends, among other things, on the dosage, route and timing of administration. So it was perhaps not suprising that total protection was not achieved but it would be very interesting to study the amount of antibody produced in response to the vaccine. It is likely that high titres of antibody were present in the serum but, as with other diseases and the situation found in clinical practice, this correlates poorly with clinical immune status. Indeed it may be that mice cannot mount a protective immune response against intravaginal trichomoniasis; and may be the same is true with humans. Nevertheless the model produced provided a good opportunity to investigate the immune response to intravaginal trichomoniasis.

Immunogenicity of T. vaginalis has already been observed and some of the antigens characterised (Alderete, 1983; Connelly 1985; Alderete and Kasmala, 1986; Garber 1986) and hyperimmune serum is relatively easily obtained (Gombosova et al., 1986). It is therefore probable, given that T. vaginalis is immunogenic, that my vaccination procedure yielded a

high titre of antibodies. It seems possible that these antibodies were not protective because the secretory immune system was not triggered effectively or that the antibodies were not directed against molecules present on the intact surface. The acquisition of soluble serum antigens by T. vaginalis has already been observed (Peterson and Alderete, 1983) and it was speculated that this is a method of evading the immune response.

It is possible that an effective secretory response can best be achieved by stimulation of the secretory system rather than systemic vaccination and that subcutaneous vaccination is particularly ineffective (Wira and Sandoe, 1987). Reports are available of oral vaccination affording protection against Herpes infection and chlamydial infections of the genital tract (Sturn and Schneweis, 1978; Nichols et al., 1978). For this reason a short experiment involving one administration, by the oral route, of freeze-thawed trichomonads was performed. This also had no effect on the infection rate. Again it would be necessary to follow up this study with measurements of antibody titre of the vaginal/cervical secretions to determine whether the immune response was not being stimulated or if a response was triggered but was not protective. This was attempted by me but results are not available.

There is a report of a study involving injection of hyperimmune serum into the cervix-uteri and vagina of

patients with trichomoniasis refractory to treatment. A number of patients, too small to be significant, reported great improvement in the clinical symptoms (Aburel et al., 1963). This kind of experiment would be very interesting to perform using the available mouse model. Other experiments involving passive immunisation would also be valuable.

Another possibility to explain the lack of protective immunity observed both in these experiments and clinical infections (Mason, 1979; Street et al., 1982) is that of active suppression of the immune response by the parasites. Other protozoan infections have been shown to stimulate suppressor T-lymphocytes (Diamanstein et al., 1980; Ferrante and Smythe, 1984) and Mason and Patterson (1985) reported that T. vaginalis stimulated a T-lymphocyte population, although its identity is not yet known, from patients with active trichomoniasis. They speculated that because a non-pathogenic amoeboflagellate Naegleria grubeŕ does not similarly stimulate mitogenic activity, then suppressor cell stimulation is an important factor in determining the pathogenic potential of protozoa. However, no difference in the response of T-cells to antigens derived from pathogenic and non-pathogenic strains of T. vaginalis was observed. It is therefore unlikely that suppression of the host response is essential to the survival of T. vaginalis.

The influence of the estradiol treatment of the mice on the effect of the immune response should also be considered. Previous studies have shown that the levels of total immunoglobulins in the female reproductive tract are under the control of the sex hormones (Wira and Sandoe, 1977). Although estrogen appears to increase levels of IgG, IgA and secretory component in the uterine lumen (Wira and Sandoe, 1980; Sullivan et al., 1983) it inhibits their levels in cervicovaginal secretions (Wira and Sullivan, 1985). Wira and Sandoe (1987) observed that rats that received Peyer's patch immunisation with sheep red blood cells showed specific IgA and IgG antibodies in the vaginal secretions. This was markedly inhibited by prior estradiol treatment. Although this may be important in the mouse model (and more work on this aspect would be very interesting) it is probably not so important in humans, as there is no clinical evidence to suggest that infection only occurs during part of the menstrual cycle. Also, given that a protective immune response does not appear to be evident, the influence of estrogen on presence of antibodies to trichomonads may be of little clinical value.

Assessment of the immune response by rechallenging mice that already resolved an infection was also attempted. There seemed to be no protective response generated by the resolution of a previous infection, even though the challenge was with the same parasite

line. Attempts to infect mice that had been intravaginally inoculated with T. vaginalis, but had not shown an infection, were also successful; many of the mice became infected. This suggests that resistance demonstrated on the first exposure to T. vaginalis was probably not innate but was either due to a slight, undetectable variation in the infection procedure, or a slight change in host physiology, probably particularly the vaginal microenvironment. Mice that have been previously subcutaneously infected are also not protected from intravaginal infections.

Other experiments that would be helpful in evaluating the immune response would involve assessing if subcutaneous infection protected mice from subsequent subcutaneous infections. Other work conducted in the laboratory indicated that this was not so (A. Bremner-personal communication). More study of different mouse strains may also provide an insight. For instance it would be useful to determine if the apparent resistance to infection of some strains was due to a more responsive immune response or less favourable vaginal environment.

Overall, the results concerning the immune response to an intravaginal infection with trichomonads, indicate that the triggering of a protective immune response in the mouse (by oral, subcutaneous or intravaginal routes) presents difficulties. As trichomonads have been shown

to be immunogenic and the vagina is capable of mounting an immune response, these results suggest that if a response was triggered then it has little therapeutic effect or that there is parasite evasion of this response. This would correlate well with knowledge of the human infection as antibodies versus T. vaginalis have been shown to be present in the majority of infected parasites but no correlation between the severity of the disease and antibody secretion could be discerned (Su, 1982). It appears, therefore, that the response is not protective.

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